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636. THE INFLUENCE OF MILK YIELD ON FERTILITY IN DAIRY CATTLE

BY ELISABETH J. CURRIE

New Zealand Dairy Board, Wellington, New Zealand

The opinion is frequently expressed by farmers and veterinarians that high-yielding dairy cows do not conceive as easily as those producing at a lower level. There has been some slight confirmation of this opinion both in New Zealand and overseas. In New Zealand, in the higher-producing herds culling for sterility has been found to be heavier (1), and there is a greater percentage of barren cows (2) than in the lower-producing herds. In Denmark, Rottensten & Andersen (3) found that herds at higher levels of production were not so fertile as those at lower levels, when fertility was measured by the percentage of cows in the herd holding to first insemination. On the other hand, Boyd, Seath & Olds (4) in the United States, reporting on cows ranked according to milk yield, found very little indication that breeding efficiency was affected by milk production. Nor did Ward (5) in New Zealand, classifying groups of herds according to average butterfat production, find any evidence that high production was associated with sterility.

In all these studies, either the cows or the herds were ranked according to their milk yield. But this has meant disregarding differences in management and feeding between the different herds. In Denmark and the United States, where feeding of concentrates according to milk production is standard practice, this may not be of much importance, but under New Zealand conditions of out-door grazing and, in most herds, no feeding of concentrates at all, the milk yield is to a marked degree dependent on climatic conditions and on management. By considering the high- and low-yielding cows within a herd the dilemma of what is a high yield in a given set of circumstances may be avoided. The present analysis has been made from this stand-point.

If indeed the ability of a cow to conceive is affected by the extent of her milk yield, then the yield may affect either the current conception or the subsequent one. In the present paper the first of these assumptions was studied. The cows in each of a number of herds in the district Herd Improvement Associations were divided into those holding to a first artificial insemination service and those not holding to first service, and their milk yields on the testing days nearest the insemination dates compared. The significance of the differences in the interactions of each of these levels of fertility with milk production between both herds and associations was also examined.

SELECTION OF THE MATERIAL

The New Zealand dairy practice of calving the great majority of cows in July and August, and the restriction of the artificial breeding season to about 2 months, from the middle of September to the middle of November, has meant that the cows used in the analysis were at much the same stage of lactation, and under fairly uniform feeding conditions. They had also the advantage of the spring flush of feed, and so most of the milk yields used,

which were those recorded in October, with a few in late September or early November, were the peak yields, or very near the peak.

There were available for the analysis 524 herds in the North Island and in the northern part of the South Island. The milk yields were recorded each month by the six Herd Improvement Associations which control herd recording in New Zealand. The term 'Association', therefore, implies a geographic distribution. The herds were those using the artificial breeding service. From ten to twenty-five cows were selected from each herd.

Certain restrictions were observed in selecting the cows in order to eliminate known causes of infertility. Cows were not chosen unless the first insemination took place at least 1 month after calving, since it is well established that the conception-rate for cows served less than 30 days after calving is much below average. In addition, those inseminated more than 3 months after calving were also passed over, as this unusually long interval between calving and mating might have been due to ill-health.

To avoid the effects of age on milk yield, only mature cows were chosen, which made the use of age-corrections unnecessary. The final restriction was that only Jersey cows were used, to avoid the influence of different breeds on milk yield.

Under these conditions, in most of the herds all the available cows were selected, but in those herds in which there were more than twenty-five suitable cows the first twenty-five in test number order were taken.

Table 1 gives a summary of the data.

Table 1. *The numbers of herds and cows used, and a summary of the information obtained*

Association	No. of herds	Total no. of cows	Percentage of cows holding to 1st service	Average milk yield recorded for month of service (lb.)	
				Cows holding to 1st service	Cows not holding to 1st service
Northland	67	1051	58·2	30·3	31·2
Auckland	203	3648	57·3	31·4	31·7
Bay of Plenty	43	825	59·4	33·3	33·7
Taranaki	103	1930	57·4	33·6	33·4
Wellington	88	1437	50·2	34·0	34·2
South Island	20	343	51·9	35·6	35·0
Total and average	524	9234	56·3	32·4	32·7

ANALYSIS OF VARIANCE

An analysis of variance procedure was designed to test for a significant difference in milk production as between the two fertility levels, holding to first service, and not holding to first service. The design also enabled a test to be made for the significance of the interactions of herds with fertility levels, and of associations with fertility levels.

The analysis was made on the difference between the mean milk yield of the two groups of cows in each herd, those that held to the first service and those that did not. Taking x_{kijt} as the milk yield in the month of service of the jth cow in the ith herd in the kth association at fertility level t($t=1$ for 'in calf' and $t=0$ for 'not in calf') the difference between means is then

$$z_{ki}=\bar{x}_{ki.1}-\bar{x}_{ki.0}.$$

The linear model for z can be taken as

$$z_{ki}=\alpha+d_k+h_{ki}+e_{ki},$$

where α is the difference between the main effects of the two fertility levels, d_k is the difference between the interactions of the kth association with the fertility levels, h_{ki} is a similar difference of interactions for the ith herd in the kth association, taken as being random with variance σ_h^2, and e_{ki} is an error term having variance σ_ϵ^2/w_{ki}. w_{ki} is twice the harmonic mean of n_{ki1} and n_{ki0}, the number of cows in the (ki)th herd holding and not holding to service respectively.

σ_ϵ^2 is the variance of the residual error term in the linear model of the x's.

By using an estimate of σ_ϵ^2 from the within-herd-and-fertility-level mean square of the x's, approximate F tests can be derived from the within-and-between associations analysis of variance of the z's. These involve two weighting coefficients, λ and μ derived from the w's.

RESULTS

Analysis of variance of z's

Term	D.F.	Mean square
Between associations	5	4·97
Between herds within associations	518	9·39

The estimate of σ_ϵ^2 obtained from the x's ($9234-1048=8186$ D.F.) was 37·78.

The values of the weighting coefficients were

$$\lambda=\frac{1}{518}\sum_k\left(1-\frac{1}{N_k}\right)\sum_i\frac{1}{w_{ki}}=0{\cdot}28,$$

$$\mu=\frac{1}{5}\sum_k\left(\frac{1}{N_k}-\frac{1}{524}\right)\sum_i\frac{1}{w_{ki}}=0{\cdot}27,$$

where N_k is the number of herds in the kth association.

Then $\dfrac{9{\cdot}39}{(0{\cdot}28)\,(37{\cdot}78)}=0{\cdot}89$, tested against $F_{518,8186}$ is not significant. This indicates that σ_h^2 is not significantly different from zero, that is, the difference in production between fertility levels is constant from herd to herd.

Similarly, $\dfrac{4{\cdot}98}{9{\cdot}39+(0{\cdot}28-0{\cdot}27)\,37{\cdot}78}=0{\cdot}53$ tested against $F_{5,518}$ is not significant. This indicates that the difference in production between fertility levels is constant over all associations.

Finally, $\dfrac{\bar{z}}{\sqrt{\dfrac{37{\cdot}74}{524}}}=0{\cdot}33$ tested against the standardized normal distribution is not significant. This indicates that the two fertility levels are not associated with the level of milk yield.

DISCUSSION

Although, as shown in Table 1, the cows not holding to first service had a slightly greater milk yield than those holding to first service, a statistical analysis showed that the difference was not significant. It is clear, then, that whether or not a cow holds to first service is unaffected by her milk yield at that time.

The other results were satisfactory evidence of homogeneity within the sample, and showed that over all associations and herds the fertility of the cow, measured by whether or not she held at first service, was not associated with the amount of her milk yield.

SUMMARY

9234 cows in 524 herds were divided into two groups in each herd, those holding to first service and those not holding to first service. The differences between the milk-yield means of each group in each herd were tested by an analysis of variance procedure, which showed no significant difference in milk production between the two fertility levels. It was also shown that the sample was homogeneous. The study shows that the amount of their milk yield during the month of service does not adversely affect the fertility of dairy cattle.

The writer wishes to thank Miss O. M. Castle, New Zealand Dairy Board, for assistance and advice in the selection of the material and Mr Shayle Searle, New Zealand Dairy Board, for designing the analysis of variance and supervising the statistical work.

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637. ACID PRODUCTION IN MILK BY STARTER CULTURES—THE EFFECT OF PEPTONE AND OTHER STIMULATORY SUBSTANCES

By ELLEN I. GARVIE and L. A. MABBITT

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(With 1 Plate and 4 Figures)

The rapid development of acid in the cheese vat is an important factor in the satisfactory making of hard cheese. Slow or arrested acid development may be due to phage infection but this can be prevented by adequate precautions during starter making and by rotation of cultures of different phage type. Penicillin in the milk has been blamed for starter slowness but difficulties from this cause with bulk milk appear to be infrequent at the present time (1). Other factors appear to be important in slow acid development; in particular, the inherent activity of the organism concerned and the composition of the milk.

Starter culture strains which, growing in milk, produce acid at a rate suitable for hard-cheese making are called 'fast' and those failing in this requirement are called 'slow'. Fast acid production is not a stable character. Daily subculture in milk will often result in an initially fast culture becoming slow. Intermediate stages can also be demonstrated. Thus it was found that of the colonies on a plate culture of a fast strain about 2% were slow when inoculated into milk (2). At present the only known method of maintaining activity in a strain is to plate out the cultures frequently. Colonies are picked and those which prove to be fast acid producers in milk are selected for continued propagation.

The differences between a slow strain and the fast strain from which it is derived are not understood; various substances, e.g. liver fraction (3), yeast extract (4) and peptone (5) when added to milk will stimulate a slow culture to produce acid at a rate equal to that of the corresponding fast strain. The increased activity only remains as long as the stimulating substances are present.

Changes in starter activity due to variations in milk quality are well illustrated by 'winter slowness'. This may or may not be overcome by heat treatment of the milk (6). There is evidence (7) that the activity of the starter may be correlated with the amount of 'peptide fraction' in the milk.

It seems clear, therefore, that slow acid production in the cheese vat may be caused by the use of a nutritionally exacting culture or by using milk which is nutritionally deficient for the starter metabolism.

A study of the stimulatory effect of peptone was made to try and clarify some of these problems.

METHODS

Cultures

Streptococcus cremoris 14, a slow variant isolated from the starter culture National Collection of Dairy Organisms 924, was chosen. The culture was propagated daily in litmus milk using a 1% inoculum of the previous 24 hr. old culture. Incubation was at 22° C.

Assay techniques

Tube assay

Two variations of the tube assay were used: the 'long' test and the 'short' test. Milk was used as medium throughout, and was taken from a single 10 gal. churn after the evening milking at the Institute farm. The sample was placed in cold store overnight and the following morning divided into the required quantities. Generally 30 ml. quantities were used, but the quantity was decreased on occasion to 10 ml. or 1 ml. The milk was autoclaved for 5 min. at 15 lb., cooled quickly and, if not used immediately, put in cold store until required.

The short test. After addition of the substances to be tested, the tubes of autoclaved milk were inoculated with 1% of an 18 hr. culture. The tubes were stoppered with rubber bungs, well shaken, and placed in a water-bath at 30° C. for 5½ hr. with mixing by inversion at hourly intervals.

The long test. Samples were inoculated in the evening from a 6 hr. culture using 1% of a $\frac{1}{100}$th dilution in ¼ strength Ringer solution. After mixing the samples were placed in the water-bath for 16 hr (i.e. overnight) without further mixing.

The long test was used with 1 and 10 ml. amounts as well as 30 ml. With both the smaller quantities single estimations of acidity only were made. In these cases the sample was titrated directly in the tube in which it was incubated.

Assessment of results. The tubes were placed in iced water and duplicate 10 ml. quantities of each sample (or in some cases the whole sample) were titrated with N/9 NaOH using phenolphthalein as indicator.

The increment in acidity was the difference between the mean of the duplicate titrations on any sample and the mean of the duplicate titrations on an uninoculated control. If the increment in acidity developed by culture 14 in milk is subtracted from the increment in acidity of the other tests, an estimate of the stimulation is obtained. The control (culture 14 in milk) varied slightly from day to day and it was considered necessary to take this variation into account when estimating stimulation.

Plate assay

Litmus milk at 45° C. was inoculated with 1% of a 24 hr. culture of 14, and after mixing with an equal volume of 4% agar in water at the same temperature was used to prepare plates. One loopful (0·01 ml.) of the test solution was placed on a 0·5 cm. disk of filter-paper (Whatman no. 40) lying on glass. When the paper disk had dried it was placed on the surface of the solidified agar. The plates were incubated at 22° C. for 24 hr.

Estimation of stimulatory substance. The diameter of the acid zone was measured and the percentage activity contained in the test solution estimated by reference to a standard curve. The standard curve was plotted from the stimulation given by known dilutions of peptone solution. The activity of 10% peptone was taken as 100%.

Standard solution

In both assay techniques a 10% solution of Evans's peptone in water was used as standard. The solution was adjusted to pH 7·0 with NaOH and sterilized in the autoclave (15 lb. for 20 min.).

For the tube assay 1 part of peptone solution was added to 10 parts of milk, giving a final concentration of 1% peptone.

Study of growth in milk

A comparison was made between the behaviour of 14 in milk and in milk containing 1% peptone. Two experiments were made. In Exp. A the inoculum was 1% of $\frac{1}{1000}$th dilution of a 24 hr. culture. Duplicate samples were inoculated; one tube was placed at 30° C., the other tube in an iced bath which was heated to 30° C. at a predetermined time during the night. By sampling both tubes a 24 hr. time period was covered. In Exp. B the inoculum was 1% of an 18 hr. litmus milk culture, incubation was at 30° C. and all observations were made on one sample.

The numbers of organisms present were estimated by the colony count method plating out serial $\frac{1}{10}$th dilutions of the milk in yeast-dextrose agar. The plates were incubated for 3 days at 30° C. For microscopic examination 0·01 ml. of $\frac{1}{10}$th dilution of the sample was smeared over 1 sq.cm. of a glass slide and stained by the method of Broadhurst & Paley (8).

Estimation of sugar. The total sugar content of a suitable dilution of the sample was determined by the anthrone method (9,10) using the reagent described by Fairbairn (11). In experiments where sugar was estimated cotton-wool was excluded at every stage of the proceedings to avoid any contamination of the sample.

RESULTS

Examination of assay methods

The tube-assay method might give a better indication of starter activity in the cheese vat than the plate assay, but in many instances either insufficient test material was available or the numbers of samples to be tested were too great for the tube assay to be used. In such cases the plate assay was very convenient. A critical comparison of the two types of assay was not made, but a few substances were examined by both methods and generally the results agreed.

A statistical examination of the short test is described elsewhere (12). Because in the present work several replicate tubes were not included slight stimulation of the culture might easily be missed when the short test was used. By changing to a lower inoculum and a longer incubation time the maximum stimulation could be conveniently observed and slight stimulation was more readily detected.

Table 1 shows the effect of decreasing concentrations of peptone as assayed by the three test methods. The tube assay was relatively insensitive to a decrease in peptone concentration between 1 and about 0·1%; slight stimulation was still detected with 0·01% peptone. With the plate assay, on the other hand, a linear response was observed as the peptone concentration was decreased from 20 to 2·5%. The stimulation faded out below 2·5%.

Separation and properties of the stimulatory fraction from peptone

Precipitation with organic solvents. The activity of the supernatant after addition of increasing concentrations of methanol, ethanol and acetone to 10% aqueous peptone are shown in Fig. 1. In no case was a sharp separation of the active substances obtained. It seemed probable that several active components were involved. The 'activity' was

completely soluble in 70% ethanol. At this concentration the precipitate obtained was light brown in colour and amounted to about 40% of the total solids. Thus nearly a two-fold concentration of the active component could be obtained by precipitation with 70% alcohol.

Table 1. *The effect of different concentrations of peptone on culture* 14

Tube assay									
Concentration of peptone soln. (%)...	2·0	1·0	0·5	0·25	0·125	0·1	0·05	0·01	0·00
5½ hr. incubation									
Increase in acidity (ml. N/9 NaOH 10 ml. milk)	—	2·15	—	—	—	2·45	—	—	0·45
16 hr. incubation									
Increase in acidity (ml. of N/9 NaOH 10 ml. milk)	—	4·23	4·7	4·625	4·15	3·15	2·275	2·425	2·39
Plate assay									
Concentration of peptone soln. (%)...	20·0	10·0	5·0	2·5	1·25				
Diameter of acid zone (cm.)	2·3	1·9	1·5	0·9	0·0	} Replicate plates			
	2·3	2·0	1·7	0·9	0·0				
	2·2	1·9	1·6	0·5	0·0				
	2·4	1·8	1·7	0·8	0·0				

Results for the tube assay are expressed as the difference between the titre for any sample and its uninoculated control.

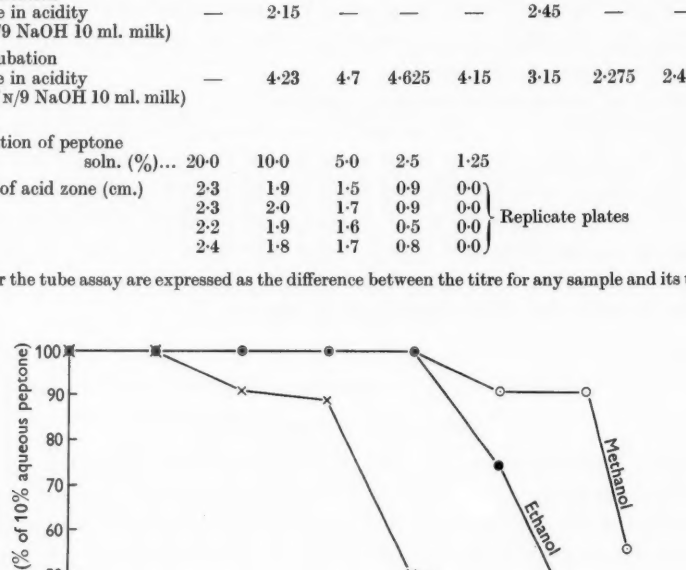


Fig. 1. Solubility of stimulatory factor from peptone in various concentrations of organic solvents.

The separation of peptone fractions on an ion-exchange column. On the assumption that the active material contained amino nitrogen it was worth while to attempt separation on Dowex 50 (×4) resin (Na^+) which is known to be capable of giving good separation of amino-acids and peptides. Two ml. of a 10% peptone solution at pH 2 was applied to the column (150 × 1 cm.) and eluted with 0·2 M citrate buffers of pH 3·4, 4·2 and finally with 2 N-NaOH. The eluate was collected in 350 2·5 ml. samples, neutralized and assayed for activity by the plate method. The activity was not detected in any sample. This

supported the view that several components were responsible for the activity and their separation resulted in the activity of individual samples being too low for detection on the plate.

Separation by paper partition chromatography. A spot of aqueous 10% peptone solution was placed on Whatman no. 1 paper (10 × 10 in.) and the chromatogram developed for 18 hr. by the ascending technique with butanol-acetic acid (13) as solvent. After drying the paper was placed on an assay plate 9 in. square to determine the position of the active compounds. A streak of activity was obtained which commenced about 5 cm. from the origin and continued almost to the position of the solvent front. The streaking may have been due to the salt content of the peptone. Desalting on a Dowex 50 (× 4) in the H^+ form did not, however, improve the separation.

When a chromatogram prepared in the same manner was sprayed with ninhydrin, a yellow brown streak in addition to discrete zones of amino-acids was revealed in the same relative position as the streak of activity on the assay plate. It suggested that the active substance was peptide in nature and had an affinity for filter-paper which prevented separation into a discrete zone with the solvent employed.

Preparation of an active peptide fraction. It had been noted that if desalting of peptone was attempted on a Dowex 50 (× 8) resin about 20% of the activity could be eluted with water, the remainder being adsorbed on the resin. Examination of the aqueous eluate by paper partition chromatography showed that free amino-acids were absent, but that material, producing a faint yellow-brown colour with ninhydrin and forming a streak down the chromatogram, was present. On a large assay plate and using a duplicate unsprayed chromatogram a streak of stimulation was observed in a position corresponding to that of the ninhydrin positive material.

Hydrolysis of this fraction for 18 hr. with 6N-HCl did not alter the activity as estimated by the plate assay. When the hydrolysate was run on a paper chromatogram the yellow-brown streak was absent, but many amino-acids were present.

The effect of hydrolysis on peptone activity. Two grams of peptone were hydrolysed for 18 hr. at 100° C. in 6N-HCl. Most of the HCl was removed by drying on a Craig evaporator (14). A plate assay of the product showed it to be equal to peptone in its stimulatory properties. These results were confirmed by a tube assay; the material coagulated milk if added in a concentration equivalent to 1% peptone, but both the hydrolysate and peptone were examined satisfactorily in milk as 0·1% solutions (acidity increases of 4·0 and 3·0 (peptone)).

When the hydrolysate was run on paper and the paper placed on an assay plate three faint localized areas of stimulation only were visible in the portions leucine-phenylalanine, valine-methionine, threonine-alanine and proline.

Stimulation by amino-acids. 0·02M solutions of twenty-two amino-acids were examined by the plate-assay method. They comprised arginine, lysine, glutamine, asparagine, aspartic acid, glutamic acid, phenylalanine, proline, tryptophane, hydroxyproline, cystine, tyrosine, glycine, alanine, valine, leucine, isoleucine, norleucine, methionine, histidine, threonine and serine. Individually no stimulation was shown. When eighteen were examined together (glutamine, asparagine, hydroxyproline and norleucine excluded) a response was obtained. The stimulation was less than with peptone and the zone of activated growth was ill-defined and could not be measured. Different combinations of amino-acids were examined but a complex picture of stimulation was found. Cystine

appeared to be most active, but in the absence of cystine other combinations of amino-acids also gave a good response. Cystine was examined by the tube assay but no stimulation was recorded.

Stimulation by other materials. Several other substances, all of which were expected to contain peptides and simple breakdown products of protein, were tested. Corn steep liquor and liver extract were both active; neither of these solutions increased the acid produced by 924, a fast variant of 14. Both liver extract and corn steep liquor appear to be more effective than peptone in stimulating acid production by 14, by the tube-assay method.

Trypsinized and acid hydrolysed casein were also both active, and weight for weight were equivalent to peptone. Trypsinized milk was not active when tested by the plate assay or added as 1% to milk, but as the amount of casein in milk is only 2–3% it is probable that failure to observe activity was due to the small concentration of the material tested.

Stimulation due to enzymes. If trypsinized casein activated 14 it was reasonable to assume that an enzyme acting directly on the casein in the milk would also be effective. The amount of amino-compounds present would be between 2 and 3%, which should be adequate to show stimulation. As expected commercial rennin solutions were found to produce a zone of stimulation by the plate-assay method. When added to milk both commercial and crystalline rennin stimulated 14 to produce acid. Presumably the enzyme(s) present attacked the casein and the products were more readily available to 14 than the nitrogen compounds naturally occurring in the milk.

It has long been known that spore-forming bacteria may stimulate acid production of other bacteria in milk (15). Milk was inoculated with *Bacillus subtilis* and incubated for 18 hr. at 37° C. and was then used for a tube-assay test with 14. Marked stimulation was observed (Table 2). Also Seitz-filtered whey extracts of milk in which *B. subtilis* had grown were found to stimulate 14 by the plate assay. When the extracts were steamed for 30 min. the activity was reduced. It is reasonable to assume that the proteolytic enzymes produced by *B. subtilis* by attacking the casein in the assay medium were responsible for the activation of 14.

The growth of 14 *in milk and milk + peptone*

Figs. 2 and 3 show the titratable acidity and colony count of cultures 14 and 924 growing in pasteurized milk (63° C. for 30 min.). When autoclaved milk was used similar curves were obtained. Both the fast strain 924 and the slow strain 14 increased rapidly in numbers in milk. The rate of increase and the maximum count were similar for the two cultures, and the growth rate of neither culture was affected by the addition of peptone. However, while 924 produced acid equally rapidly in milk and milk with added peptone, 14 only showed a similar activity when peptone was present. Thus the addition of peptone to 14 when growing in milk produced an increase in acid production without affecting the growth rate. A second slow starter culture 275 gave results similar to those with 14. However, in the tube assay 'short test' and with peptone present 14 produced less acid than 924 (a difference of 0·65)—suggesting that even the addition of a large quantity of peptone to the cheese vat would not overcome slowness if 14 was used as starter.

A second experiment (B) was planned to study the utilization of lactose by 14. The results are given in Fig. 4, and they show that although the culture was increasing in

numbers in milk it was not using lactose, or using it very slowly. With the addition of peptone to the milk the quantity of lactose utilized was much increased.

Smears were prepared from culture 14 growing in milk with and without peptone. Microscopic examination showed no visible difference in the size of cell or in the amount

Table 2. *Stimulation of culture* 14 *by substances other than peptone*

Substance	Concentration of test solution* (%, w/v)	Tube assay: Short test stimulation (ml. NaOH/10 ml. milk)	Tube assay: Long test stimulation (ml. NaOH/10 ml. milk)	Plate assay, % activity (10% peptone = 100)
Liver extract	10	0·55 (0·45)	—	—
Corn steep liquor	10	0·85 (0·45)	—	100
	1	0·15 (0·35)	—	—
Rennin (crude)	—	—	3·15 (2·9)	—
Rennin (crystalline)	—	—	2·0 (2·9)	—
Commercial rennin	—	—	—	200
Trypsin	0·18	—	—	0
	0·5	—	—	37
Bacillus subtilis				
Whey (filtered)	—	—	1·65 (1·85)	50
Whey (steamed)	—	—	0·85 (1·85)	0
Active *B. subtilis*	—	—	2·75 (3·25)	—
Acid hydrolysed peptide fraction	10	—	—	100
Acid hydrolysed peptone	10	—	—	100
	1	—	0·9 (1·65)	—
Acid hydrolysed casein	10	—	—	100
	1	1·1 (1·7)	1·4 (1·65)	—
Casamino acids	10	0·35 (0·35)	2·8 (2·4)	—
	1	0·2 (0·35)	1·9 (2·4)	—
	0·1	0·15 (0·35)	1·05 (2·4)	—
Tryptic digest of casein	10	—	1·9 (1·85)	—

The figures in parentheses are the stimulation given by 10% peptone solution, on the same day as the test material.

* For the tube assay 1 part of the test solution was added to 10 parts of milk i.e. the concentration in the assay tube was one-tenth of the figure given above.

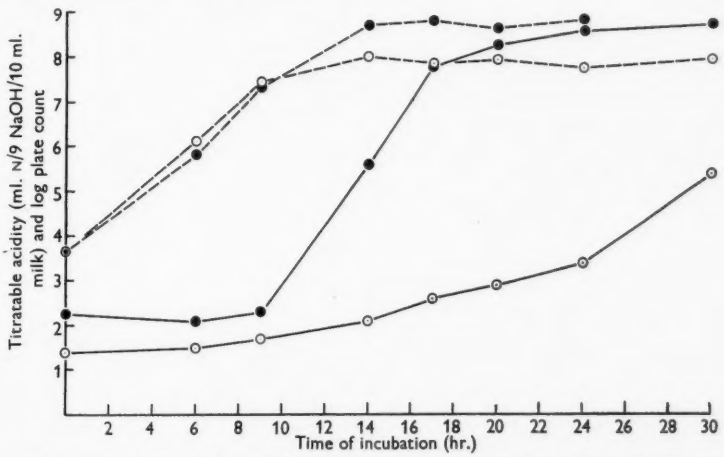


Fig. 2. Growth curves and titratable acidity of *Streptococcus cremoris* 14 in milk and milk with peptone. ○——○, acidity in milk; ●——●, acidity in milk with peptone; ○- - - -○, growth curve in milk; ●- - - -●, growth curve in milk with peptone.

of chaining and clumping. In both media the cells were oval, but while the long axis was at right angles to the chain when the culture was grown in milk, it was along the chain when peptone was present (Pl. 1A, B).

When 924 was grown on either milk agar or on yeast-dextrose agar it was not possible to distinguish the slow colonies from the fast, by colonial appearance.

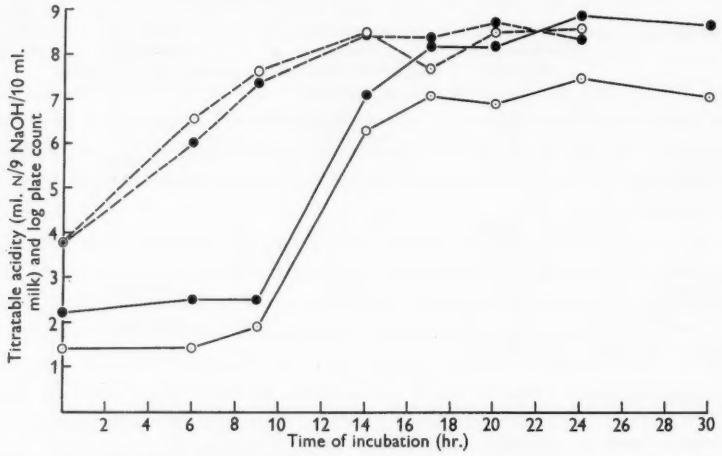


Fig. 3. Growth curves and titratable acidity of *Streptococcus cremoris* 924 in milk and milk with peptone. ○——○, acidity in milk; ●——●, acidity in milk with peptone; ○- - -○, growth curve in milk; ●- - -●, growth curve in milk with peptone.

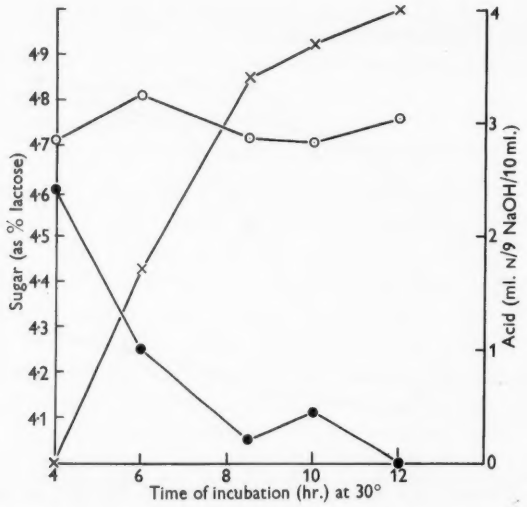
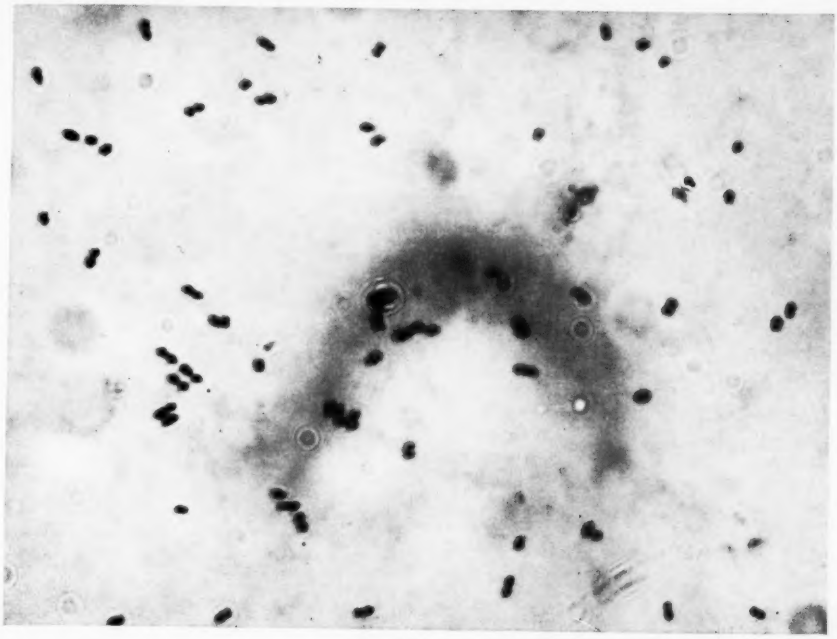
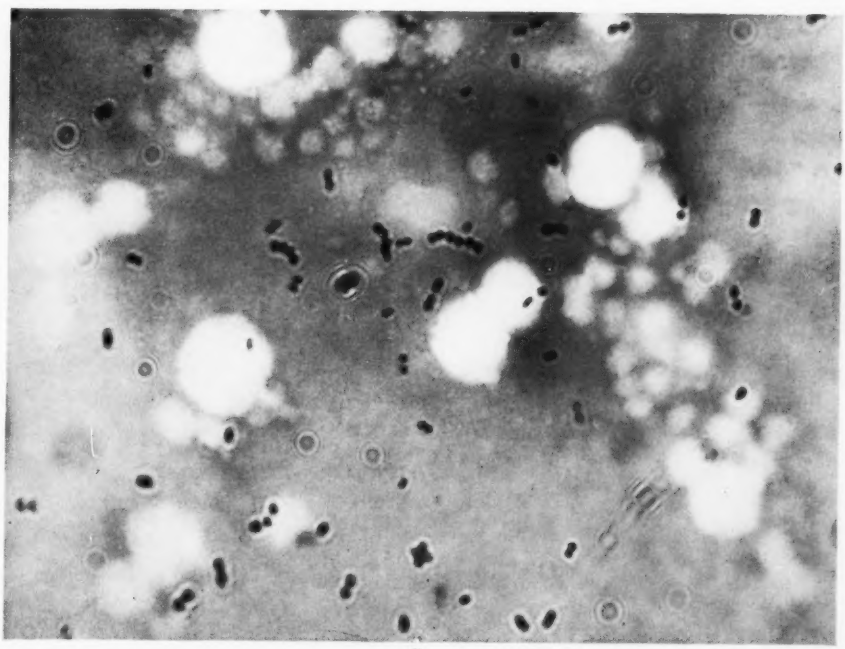


Fig. 4. Acid production and utilization of lactose in milk and milk with peptone by *Streptococcus cremoris* 14. ○——○, amount of sugar in milk; ●——●, amount of sugar in milk with peptone; ×——×, stimulation in milk with peptone.



(A)



(B)

Streptococcus cremoris **14.** 4 hr. culture in (A) milk, (B) milk with peptone.

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(*Facing p.* 312)

DISCUSSION

The results reported show that acid production by the slow variant 14 can be temporarily stimulated by the addition of peptides and/or amino-acids, and these can be obtained from casein by acid or enzymatic hydrolysis. Therefore milk contains all the necessary substances for rapid acid production by a slow starter if the milk protein is made more available by degradation.

While it is not difficult to obtain slow variants from a fast starter, no reports of a slow starter developing fast variants have been found. The irreversible nature of the change fast → slow, and the resultant failure to utilize milk protein, suggests that the loss of a constitutive proteolytic enzyme is involved. This view is supported by the previous observations that fast starters are more proteolytic than slow (2) and that slow starters have an increased activity in milk with a high peptide fraction (7).

Some strains of lactic streptococci readily lose the ability to ferment lactose (16,17), but apparently not other carbohydrates. The slow-starter cultures studied could still ferment lactose in milk, and an increased rate of acid production was not obtained by adding small quantities of either glucose or galactose. It is well known that the fermentative ability of an organism is readily influenced by the presence of a suitable nitrogen source so that if an organism loses its ability to utilize the nitrogen substrate it is not surprising that its fermentative ability is decreased. The present case is an example of this behaviour; 14 breaks down lactose only slowly in milk, but the rate of utilization of the carbohydrate is greatly increased by the addition of peptone. However, both fast and slow strains increase in numbers at the same rate in milk, and growth rate is unaffected by the addition of peptone. Similar changes in bacterial metabolism by the addition of yeastrel to cultures without affecting total numbers has been reported previously (18).

Stimulation of slow starters will presumably occur with the addition of any source of utilizable nitrogen including the protein breakdown products of the fast cells. Any starter containing a preponderance of fast over slow cells will appear fast, because the slow variants, utilizing the breakdown products of the fast cells, will produce acid rapidly. However, as the proportion of fast cells decreases there will be less stimulation of the slow and the result will be decreased acid production.

It may be that the problem of 'winter slowness' (6,19) is also linked with the availability of the nitrogen in the milk to the cultures concerned. A few preliminary observations indicate that winter slow starters are also stimulated by the addition of peptone.

A method of propagating starter strains which prevented a loss of activity would be a great advantage to the dairy industry. The problem at present appears to be to prevent an irreversible and undesirable change taking place; it is complicated by the fact that both types of cells are multiplying at the same rate and that the fast cells are themselves stimulatory to the slow. Furthermore, the slow cells are not adversely affected by the acid produced by the fast. A study of other possible differences in metabolism between the two types of cells and an investigation of their associative growth under various conditions might lead to the development of a medium which would selectively inhibit the slow variant. For the moment an empirical solution is to freeze-dry active starter strains. The starters are then kept active by using a fresh ampoule at frequent intervals.

SUMMARY

The rate of acid production by a slow variant of *Streptococcus cremoris* in milk was raised to that of the fast parent strain when peptone or acid hydrolysed peptone were added. Lactose utilization was similarly affected. However, the stimulation took place without any change in the rate of growth. Other sources of available nitrogen gave similar results.

It is concluded that the change from a fast to a slow culture which occurs on continued transfer is due to the loss of ability to utilize the nitrogen compounds of milk.

The authors wish to thank Miss Shelagh L. Gibbs for technical assistance, and Dr N. J. Berridge for the sample of crystalline rennin.

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576.851.214

638. THE INFLUENCE OF CULTURAL CONDITIONS ON THE CHARACTERISTICS OF *STREPTOCOCCUS CREMORIS*, STRAIN HP

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(With 1 Plate)

Strain HP of *Streptococcus cremoris* was isolated from a mixed culture cheese starter in 1935, during investigations at two New Zealand cheese factories, viz. Hopelands and Papatawa. The initial letters of the two names were used as the designation of the culture. HP proved to be a most active and satisfactory cheese starter; it has been in general use in New Zealand cheese factories ever since its isolation, and it is well known overseas both as a starter and as a type strain of *Str. cremoris*. Jago (1) observed, though, that HP belonged to the group of 'winter slow' starter cultures, and work at the N.I.R.D. supports this observation. Most of the early observations on bacteriophage as a cause of starter failure were made with HP as the substrate organism.

In 1937 one of us (H.R.W.) visited the National Institute for Research in Dairying and left there several cultures of streptococci including HP. Since that time HP has been kept independently at N.I.R.D. and at the Dairy Research Institute (N.Z.), under rather different cultural conditions in the two laboratories. During a visit to N.I.R.D. in 1955 by one of us (H.R.W.) it was considered of interest to examine the two cultures of HP side by side in order to determine what changes (if any) in character had taken place over the course of 18 years as a result of the different conditions of culture. A freeze-dried culture of HP was air-mailed from New Zealand and, after revival, was labelled HP (D.R.I.). The Reading culture was labelled HP (N.I.R.D.).

CONDITIONS OF MAINTENANCE OF THE CULTURES

HP (N.I.R.D.)

From 1937 to 1945 the culture was grown in chalk-milk, incubated at 30° C. until clotted, stored at 4° C. and subcultured monthly. From 1945 onwards it was grown in glucose lemco broth at 30° C., freeze-dried, stored at room temperature, and revived and redried approximately every 2 years. Over the course of the 18 years the culture was occasionally plated for purity on yeast glucose agar, but no special selection of any one type of colony was made.

HP (D.R.I.)

From 1937 onwards the culture was subcultured daily in sterilized skim-milk and incubated at 22° C. Other cultures in chalk-milk and in the freeze-dried state were kept as reserves, but the skim-milk culture transferred daily was the master culture. At

intervals of approximately 6 weeks the culture was streaked on yeast-whey agar or (in later years) lactose-yeast-phosphate agar (2) and incubated for 2 days at 22° C. Fifteen or twenty colonies were then picked at random into milk and one of the most active acid producers was taken as the new master culture.

The two main differences in the conditions under which HP was maintained in the two laboratories were therefore:

(*a*) HP (N.I.R.D.) was usually incubated at 30° C., whereas HP (D.R.I.) was always incubated at 22° C.

(*b*) HP (D.R.I.) was repurified frequently and regularly with a deliberate selection of one of the more active colonies, whereas HP (N.I.R.D.) was repurified less frequently, and a colony picked at random: moreover, HP (N.I.R.D.) had been kept in the freeze-dried state since 1945.

EXAMINATION AND COMPARISON OF THE CULTURES

Morphology

HP shows a characteristic morphology when a culture is grown in skim-milk at 37° C. for 5–6 hr., and then examined under the microscope after staining with methylene blue. The exposure to 37° C., almost the limiting temperature for growth, induces in most strains of *Str. cremoris* the appearance of involution forms which, with some strains, are sufficiently characteristic to serve as a means of differentiating one strain from another. Under these special conditions HP gives medium length chains (15–25 units) with the distinguishing feature that the terminal cocci tend to be swollen to about double the normal size. HP (N.I.R.D.) and HP (D.R.I.) both showed the characteristic swollen terminal cocci, but there was a difference in appearance between the cultures in that HP (N.I.R.D.) gave longer chains of smaller cocci than did HP (D.R.I.). The difference was constant and distinctive enough to permit ready identification of the cultures under the microscope. Pl. 1 shows the typical appearances of the cultures.

Biochemical reactions

Both cultures gave the reactions considered typical for *Str. cremoris*. They fermented glucose, fructose and lactose, and failed to ferment maltose and dextrin. They grew at 10° C. and failed to grow at 40° C. They were inhibited by 4% NaCl and by 2% bile salt. The main differences between them were that HP (D.R.I.) was more liable than HP (N.I.R.D.) to inhibition by methylene blue in milk at 30° C. and also was rather slower to reduce and clot litmus milk. However, the differences were not significant.

Serological reactions

The cultures, after growth in glucose lemco broth overnight at 30° C., were spun down and extracted with HCl in the usual way. Incidentally it was noted that HP (D.R.I.) gave a flocculent growth in broth, whereas HP (N.I.R.D.) gave a more diffuse and less heavy growth. The extracts were tested against group sera prepared in rabbits against group N, D and E streptococci (3). Both the HP extracts gave typical group N reactions and showed no precipitation with group D and E sera.

Type sera were prepared by inoculating rabbits with heat-killed suspensions of the organisms (4), a series of four injections at 3- to 4-day intervals being given. The two sera

A

B

A. *Str. cremoris* HP (D.R.I.): ×2000.
B. *Str. cremoris* HP (N.I.R.D.): ×2000.

(*Facing p.* 316)

gave agglutination with both cultures to a titre of more than 1/1280 overnight at 55° C. In both sera HP (N.I.R.D.) gave finer flocculi than did HP (D.R.I.). In serological reactions the cultures were thus identical.

Phage reactions

A phage which normally appears in cheese whey wherever culture HP is used as a cheese starter in New Zealand was tested against the two cultures by making a series of tenfold dilutions in saline and placing drops of the dilution on mats of cultures on agar plates. The phage acted readily on both organisms but to a slightly higher titre (one extra dilution) on HP (D.R.I.) than on HP (N.I.R.D.). The slightly higher sensitivity of HP (D.R.I.) to the phage was evident with phage preparations made with the use of either organism as the substrate. Three other phage races which act upon HP (D.R.I.) also acted on HP (N.I.R.D.) in each case to a slightly lower titre. There was thus evidence that the cultures showed the same phage pattern although with a curious and quite regular slight difference in sensitivity.

Activity as cheese starter

After the above tests were carried out in England, freeze-dried preparations of the two cultures were sent by air-mail to New Zealand where better facilities were available for making a comparison of them as cheese starters. The two cultures were used as cheese starters in two vats, each containing 70 gal. of milk from the same bulk supply. Good quality Jersey milk (4·9% fat, 2·73% casein) was flash pasteurized at 150° F. (65·6° C.), cooled to 88° F. (31·1° C.) and inoculated with $1\frac{3}{4}$% of the appropriate starter. An attempt was made to dry and to salt both vats at the same acidities. The comparison between the cultures could thus be expressed as differences in the time needed for the two parts of the cheese-making process. The results are set out in Table 1.

Table 1. *Cheese-making details, using HP (D.R.I.) and HP (N.I.R.D.) as starters*

	1st day, 24. ii. 56		2nd day, 5. iii. 56	
	HP (D.R.I.)	HP (N.I.R.D.)	HP (D.R.I.)	HP (N.I.R.D.)
Acidity at drying	0·165	0·160	0·155	0·155
Time renneting to drying	2 hr. 55 min.	3 hr. 0 min.	2 hr. 45 min.	3 hr. 0 min.
Acidity at salting	0·80	0·80	0·83	0·79
Time drying to salting	2 hr. 15 min.	2 hr. 50 min.	2 hr. 40 min.	3 hr. 0 min.

The results indicate that HP (N.I.R.D.) was significantly less active in acid production than HP (D.R.I.). When examined after 2–3 weeks at 55° F. (12·8° C.) the cheeses were quite normal good quality cheeses with no significant difference between them.

DISCUSSION

Strain HP appears to be very stable in its characteristics when kept under different cultural conditions. In spite of the fact that one of the cultures had not been repurified with the specific intention of preserving its acid-producing activity it had decreased only slightly in activity in the cheese vat over the period of 18 years. Experience in New Zealand indicates that some strains of *Str. cremoris* are not as stable as HP, either in acid-producing activity or in morphology. The regular plating and selection of colonies practised at the Dairy Research Institute (N.Z.) has proved essential in maintaining the activity

of some starters. The deviations which HP (N.I.R.D.) shows from HP (D.R.I.) after such a long time under different cultural conditions are the very minor ones of slight difference in morphology, in habit of growth in broth, in type of agglutination, in sensitivity to methylene blue and to phage action, and in the already mentioned acid-producing activity. All these differences in degree are concerned with what are generally recognized as 'superficial' properties of the bacterial cell. The two cultures had retained identical antigenic properties.

SUMMARY

Cultures of a strain of *Streptococcus cremoris* HP after maintenance in two countries under different conditions preserved their main characteristics unchanged for 18 years.

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639. SOME BACTERIOLOGICAL ASPECTS OF COMMERCIALLY STERILIZED MILK

I. INCIDENCE AND NATURE OF SPOILAGE

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(With 6 Figures)

1. INTRODUCTION

The consumer demand for sterilized milk has increased during the last three decades. In some districts, notably Birmingham and the Black Country, a large proportion of the liquid supply is now distributed as sterilized milk and much of the milk produced in the West Midlands finds a market in that form. It seems that the demand for this type of liquid milk will expand further in the future and problems associated with its keeping quality will continue to be of importance. Sporeforming organisms are the only group of bacteria which may contaminate the milk during production at the farm and which can survive the heat treatment during processing.

It was thought that a study of the defects of the final product due to bacterial growth might indicate what advice on methods could best be given at the farm to ensure production of milk most suitable for obtaining sterilized milk of high-keeping quality.

When this investigation began, little published work on the keeping quality of sterilized milk in this country was available. Hiscox & Christian (1) in 1931 were the first to draw attention to the fact that 'sterilized' milk is rarely sterile. They also reported on 'carbolic' taint and discussed methods for controlling it.

In 1943, Morgan (2) pointed out the importance of sterilized milk spoilage in industrial areas of Yorkshire. He referred also to spoilage caused by thermophiles. Published information on the problem of thermophilic spoilage was extended by Clegg (3).

The work reported here commenced in June 1952, when samples of commercially sterilized milk were obtained regularly from fifteen dairies in or near Birmingham and Wolverhampton operating the commoner types of sterilizing plant.

During the period of this work, an interesting study by Burton, Akam, Thiel, Grinsted & Clegg (4) on large-scale operational runs of a batch sterilizing plant became available, indicating the effect of varying the process on the survival of sporeformers and on subsequent spoilage. The importance of more effective sterilizing conditions was emphasized.

Ridgway (5) examined over a 4-year period the keeping quality of milk sterilized by a continuous process and he showed marked seasonal variations in the spoilage of the finished product. Later, in attempting to eliminate or reduce spoilage, Ridgway (6) investigated the relationship between the spore content of the incoming raw milk and keeping quality after sterilization.

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2. EXPERIMENTAL METHODS

(1) *Investigation of incidence, rate and type of spoilage*

The sample always consisted of an unopened one pint bottle of commercial sterilized milk with crown-cap closure and was tested as soon as possible, usually within 5 hr. of processing.

The samples were opened with aseptic precautions which included swabbing the outside of the cap and neck of the bottle with methylated spirits, followed by prolonged heating of the cap and neck in the Bunsen flame. The crown cap was then removed and immediately replaced by a sterile cotton-wool plug.

Ridgway (6) has shown that the partial vacuum in bottles of sterilized milk does not prevent the development of spoilage, and consequently it was concluded that spoilage after opening is similar to that which would have occurred had the unopened bottle been held under conditions favourable to bacterial growth.

As the number of sporeforming bacteria present in commercial sterilized milk would be expected to be low, direct plating techniques to estimate initial contamination were not attempted.

From June 1952, 100 and 50 ml. quantities of the sample were poured rapidly in duplicate into 6 oz. flat wide-mouthed bottles containing sterile litmus solution. The amount of litmus solution was previously determined so that the final colour after adding the various quantities of milk would be similar. Although it was appreciated that the dye may slightly inhibit growth, it was of value in indicating slight changes in the milk under test before spoilage would otherwise have been apparent.

Two 10 ml. quantities of the sample were pipetted into McCartney bottles containing sterile litmus solution, and six 1 ml. quantities were pipetted into tubes containing 10 ml. of litmus milk medium.

The litmus milk medium was prepared from separated milk from one farm, known to have a low spore content and was sterilized by steaming for $\frac{1}{2}$ hr. on three successive days. Between each steaming all tubes were held first at 63° C. for 8 hr. and then at laboratory temperature until the next treatment. Finally, all tubes of litmus milk were incubated at 30° C. for 4 days before use. By this treatment all contamination was excluded from the medium.

One 100 ml., one 50 ml., one 10 ml. and three 1 ml. quantities were incubated at 30 and 63° C. to detect spoilage due to mesophilic and thermophilic organisms respectively. Samples were inspected daily for 7 days at 63° C. and for 28 days at 30° C., and the rate and type of spoilage was recorded.

From March 1955, the most probable numbers of mesophilic and thermophilic organisms in 100 ml. of sterilized milk were determined, using one 50 ml., five 10 ml. and five 1 ml. quantities of the sample and calculating the results from the probability tables of McCrady (7). In November 1955, incubation at 30° C. was replaced by 24° C. as this temperature is nearer the higher limits of domestic storage likely to be encountered in this country.

(2) *Isolation of cultures*

Samples showing spoilage, selected at random, were plated using yeastrel milk agar (Y.M.A.). Dilutions of 10^{-5}, 10^{-7} and 10^{-9} were usually used and plates were incubated at

30° or 63° C. Plates incubated at 63° C. were stacked in air-tight tins to reduce 'drying-out' of the medium.

Colonies, picked at random from these plates, were inoculated into litmus milk and incubated at the appropriate temperature. This process of plating and picking into litmus milk was repeated three times to ensure purity.

Plating was preferred to streaking for the isolation of pure cultures since spreaders developed less readily and isolated colonies were usually obtained.

Cultures were stored on Y.M.A. slopes in the cold store at about 4° C. for further examination.

(3) *Examination of cleansed bottles used for sterilized milk immediately prior to filling*

Cleansed bottles from seven dairies were selected before reaching the filler, and immediately plugged with sterile rubber stoppers to investigate the contamination present at the time of filling. Examination of the efficiency of the actual bottle washing and cleansing plant was not intended. Bottles were tested in the laboratory within 4 hr. of sampling. 20 ml. of $\frac{1}{4}$ strength Ringer's solution was added to each bottle, and the bottle rotated 12 times to wet all of the internal surface. The bottle was left to stand on the bench for 15 min. and then rotated a further 12 times.

12 ml. of rinse was added to 24 ml. of sterile separated milk, and three 5 ml. quantities were plated, using Y.M.A., to indicate the total contamination present. The remaining 21 ml. quantity of rinse-milk mixture was heated in a water-bath at 85° C. for 20 min., after which it was cooled rapidly. A further three 5 ml. quantities were then plated with Y.M.A., to indicate the extent of contamination by heat-resistant organisms. Plates were incubated at 30° C. and counts made after 3 days.

Litmus solution was added to the remaining 6 ml. of rinse-milk mixture, to indicate the presence of heat-resistant organisms capable of producing spoilage in milk. The mixture was incubated at 30° C. and examined for spoilage after 3, 7 and 14 days. If spoilage were present, a Y.M.A. slope was inoculated, incubated at 30° C., and a microscopic examination made when the organisms responsible for spoilage had grown.

3. RESULTS

I. *Mesophilic sporeformers*

(1) *Incidence and seasonal variation of spoilage*

Throughout, 'summer' has been regarded as the 6 months from 1 May to 31 October.

The frequency, rate and type of spoilage in the 100 ml. quantities were examined for indications of seasonal variation. Fig. 1 indicates that more samples show a higher level of contamination in winter. Spoilage under comparable temperature conditions is slower in summer (Fig. 2). It will be seen (Fig. 1) that spoilage is at a relatively high level throughout the year, and the suggestion of Mossel & Drion (8) that absence of spoilage in seven bottles selected at random should constitute an acceptable batch would clearly be an impractical procedure in this country under present conditions.

In order to examine the frequency of spoilage in the remaining 50 ml., 10 ml. and three 1 ml. quantities, assessment of spoilage was divided into two contamination levels, since the quantities selected did not permit calculation of the most probable numbers. Based on the tables of McCrady, level I included samples with low or no contamination, and

level II high contamination. The former was shown to correspond approximately to a level of less than 10 sporeformers/100 ml. of milk. The results presented in Fig. 3 confirm the seasonal trend already shown (Fig. 1), i.e. higher levels of contamination in winter.

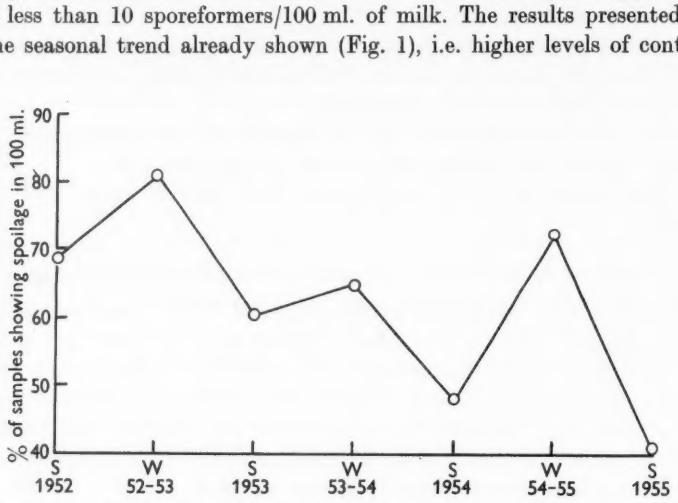

Fig. 1. Incidence of spoilage at 30° C. in 100 ml. 439 samples examined. S, summer months; W, winter months.

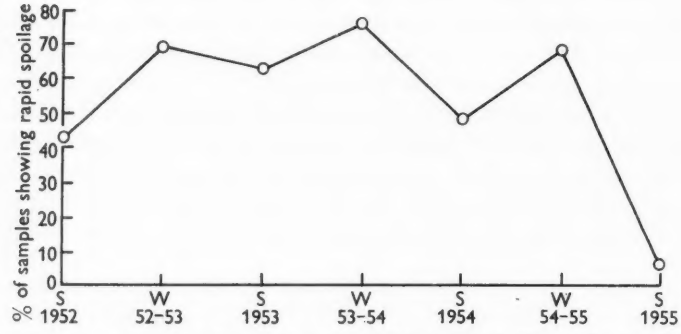

Fig. 2. Incidence of rapid spoilage (< 5 days) at 30° C. in 100 ml. 439 samples examined. S, summer months; W, winter months.

Individual dairies. Similar seasonal trends are also evident when the results from individual dairies are examined. Typical examples for two dairies are given in Fig. 4*a*, *b*. The processing conditions at these dairies may be compared:

	Processing conditions (approx.)		Samples with low level of contamination	
Dairy	Time (min.)	Temperature (° F.)	Summer (%)	Winter (%)
P	40	220	50	21
D	40	230	77	56

Dairy D, using a higher temperature, produced more samples showing a low level of contamination than dairy P, both in summer and winter.

(2) *Most probable numbers*

From March to October 1955, the probable number of mesophiles in 100 ml. of milk was calculated.

Counts ranged from 1 to 180+/100 ml. and the distribution is shown in Table 1.

Results indicate that the level of contamination is generally low, although they refer mainly to the summer period, when contamination is known to be at a lower level.

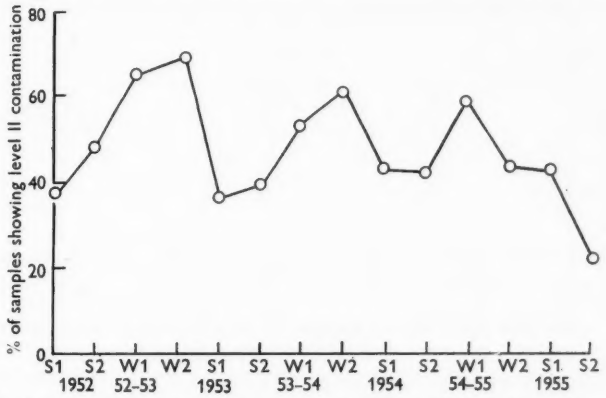

Fig. 3. Incidence of high levels of contamination (level II) at 30° C. 449 samples examined. S1, May–July; S2, August–October. W1, November–January; W2, February–April.

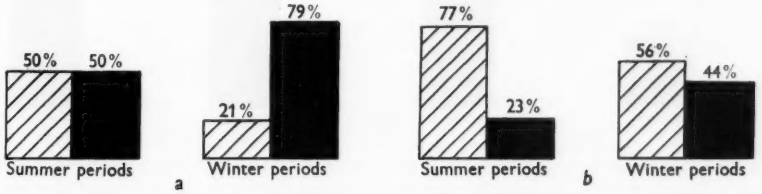

Fig. 4. (*a*) Dairy P. Seasonal variation in contamination at 30° C. (*b*) Dairy D. Seasonal variation in contamination at 30° C. ▨, % of samples showing level I contamination; ■, % of samples showing level II contamination.

Table 1. *Probable numbers of mesophilic sporeformers in* 100 *ml.*

M.P.N./100 ml.	No. of samples
0	31
1–5	20
6–10	1
11–20	3
21–50	1
51–180+	1
Total samples	57

II. *Thermophilic sporeformers*

(1) *Incidence and seasonal variation*

Data obtained at 63° C. were treated in a similar way to that at 30° C. and clear seasonal trends in the incidence of thermophilic sporeformers are apparent (Fig. 5). Thermophilic contamination, like mesophilic, is at a higher level in winter than in summer.

A comparison of Figs. 3 and 5 shows that thermophilic contamination is generally at a considerably higher level than mesophilic.

Coagulation at 63° C. occurred rapidly (usually 1–2 days), and it has not therefore been possible to detect any seasonal variations in the rates of spoilage.

Individual dairies. Similar seasonal trends are also apparent when the results from individual dairies are examined, and are shown in Fig. 6*a*, *b*.

It will be seen that the large majority of samples from these two dairies showed a higher level of contamination in winter.

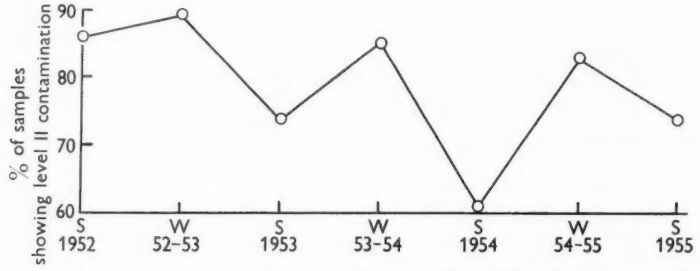

Fig. 5. Incidence of high levels of contamination (level II) at 63° C. S, summer months; W, winter months.

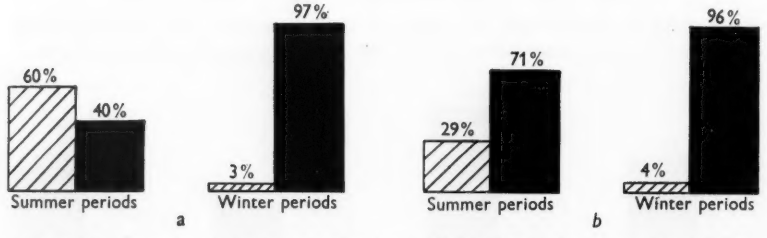

Fig. 6. (*a*) Dairy M. Seasonal variation in contamination at 63° C. (*b*) Dairy N. Seasonal variation in contamination at 63° C. ▨, % of samples showing level I contamination; ■, % of samples showing level II contamination.

(2) *Most probable numbers*

From March to October 1955, the probable number of thermophiles in 100 ml. of milk was calculated.

Counts ranged from 0 to 180+/100 ml., and the distribution is shown in Table 2. It will be seen that high levels of thermophilic contamination often occur, even though results refer mainly to the summer period, when contamination is known to be at a lower level.

Table 2. *Probable numbers of thermophilic sporeformers in* 100 *ml.*

M.P.N./100 ml.	No. of samples
0	8
1–5	15
6–10	6
11–20	6
21–50	6
51–180+	12
Total samples	53

III. *Comparison of levels of thermophilic and mesophilic sporeformer contamination*

Throughout the year high levels of contamination by thermophilic sporeformers occur more frequently than high levels of contamination by mesophiles, as a comparison of Figs. 3 and 5 indicates. Table 3 shows the relative incidence of thermophilic and mesophilic contamination in summer and winter.

Table 3. *Comparison of levels of thermophilic and mesophilic contamination*

	Total samples	No. showing level I contamination	T:M*	No. showing level II contamination	T:M*
Summer					
Thermophiles	206	51	1:2·4	155	1:0·5
Mesophiles	206	122		84	
Winter					
Thermophiles	204	28	1:2·9	176	1:0·7
Mesophiles	204	81		123	

* T:M = ratio of thermophilic to mesophilic contamination.

IV. *Contamination in bottles immediately prior to filling*

(1) *Mesophiles*

Two hundred and fifty-one cleansed sterilized milk bottles were examined, and the results are presented in Table 4. Contamination of the cleansed bottles was often high, but usually heat-resistant organisms only constituted a small proportion of the total.

Table 4. *Contamination of cleansed sterilized milk bottles*

Dairy	Bottles tested	Mean count per bottle at 30° C. Before heating	Mean count per bottle at 30° C. After heating	Samples showing spoilage in litmus milk†	Rate of spoilage at 30° C. Days <3	Days 4–7	Days over 7	Sporeformers isolated Group 1	Group 2
C	59	315	29	18 (31%)	13	2	3	14	4
A	54	121	47	11 (20%)	7	2	2	10	1
M*	46	183	76	12 (26%)	8	3	1	10	2
N	31	3418	<1	6 (19%)	3	2	1	6	0
B	30	149	7	8 (27%)	5	1	2	7	1
P	16	2	3	3 (19%)	1	1	1	3	0
Q	15	316	3	1 (7%)	1	0	0	1	0
Total	251			59 (24%)	38	11	10	51	8

* Bottles were washed on the day prior to use, stored in the inverted position and samples taken shortly before filling.

† As only 2 ml. of the original rinse was examined in this way, these figures may be appreciably lower than the actual number of bottles which contained sporeformers.

Although the number of heat-resistant organisms was generally small, they were present in 24% of the bottles examined.

Microscopic examination of the litmus milk showed that the majority of the heat-resistant organisms producing spoilage belonged to the morphological group 1 of Smith, Gordon & Clark (9) and resembled *Bacillus subtilis*. Spoilage of litmus milk at 30° C. was often rapid, and digestion predominated.

Occasionally, colony counts were higher after heating the rinse-milk mixture than before, which may have been due to the accelerating effect of sublethal heat on spore

germination, described by Evans & Curran (10). Mild heating in evaporated milk of spores of group 1 sporeformers was shown by them to hasten subsequent germination.

Results indicate that cleansed milk bottles are an important source of mesophilic sporeformers able to produce spoilage in milk.

(2) *Thermophiles*

Sixty-two bottles were examined for the presence of thermophiles producing spoilage in litmus milk and 6 ml. of a heated rinse-milk mixture, as described for mesophiles, were incubated at 63° C. and examined daily for 1 week. The rinses from fifty-one bottles showed no indication of contamination by thermophiles. Ten bottles contained thermophiles which reduced litmus milk without coagulation, and one bottle contained thermophiles which produced a firm acid clot.

It appears that cleansed milk bottles are not a major source of the thermophilic organisms which are present in sterilized milk.

4. DISCUSSION

I. *Seasonal variation*

The level of mesophilic and thermophilic sporeformer contamination in commercial sterilized milk is higher in winter than in summer. Ridgway (5, 6) suggested that aerial contamination of raw milk from dusty hay and fodders during the period when the cattle are housed is responsible. He found that raw milk had a higher spore content in winter and that the spore count of the incoming raw milk was directly related to the keeping quality after sterilization.

It is not uncommon for the processing times and/or temperatures to be increased in summer to reduce spoilage which would otherwise increase because of storage at the higher atmospheric temperatures. The lower incidence of contamination in the hot summer of 1955 (Figs. 1, 3) may be explained by such additional heat treatment. This practice may also be partly responsible for the seasonal variations of contamination which were observed, although such variations are apparent when the conditions of processing remain constant (5). It seems probable, therefore, that varying conditions at the farm and at the dairy are responsible for the seasonal trends in contamination.

II. *Most probable numbers*

Burton *et al.* (4) examined a small number of milk samples from six experimental operations of a batch sterilizer after treatment at 100° C. for 90 min. or at 113° C. for 35 min. After the less severe treatment (100° C./90 min.) mesophilic counts of 0–13/100 ml., and thermophilic counts of 0–600/100 ml. were obtained. They noted that the incoming milk contained few mesophiles or thermophiles, but the level of the latter increased considerably during passage through the plant.

Ridgway (6), investigating milk processed at 105·5° C. for 35 min. in a continuous automatic sterilizer, found mean counts of mesophiles of less than 1/100 ml. in summer and nearly 5/100 ml. in winter. Mean thermophilic counts of about 10/100 ml. in summer and 80/100 ml. in winter were obtained.

These levels are of the same order as those reported in the present work. The level of

mesophilic contamination is often low, but it is not necessary for large numbers of organisms to be present to cause spoilage, providing the temperature of the sterilized milk is favourable for growth.

III. *Contamination of bottles*

Contamination of bottles at the time of filling may be heavy, but after heating for 20 min. at 85° C., it is considerably reduced (Table 4). The commercial sterilizing process can be expected to reduce the level of contamination from the bottles even further, but some sporeformers from the cleansed bottles must be expected to survive, as they are of the same type as those which can commonly be isolated from the finished product (Candy & Nichols (11)). Although the main contamination was probably present when the bottles left the washer, some could have been added from the air. In view of the narrow bottle necks and the usually short time between leaving the washer and filling, aerial contamination, although variable, will probably be slight.

Hobbs & Wilson (12) have also reported aerobic sporeformers in washed bottles, and in bottles sampled immediately after leaving the detergent section of the washing machine. Recirculated rinse waters were also found to contain sporeformers. Vindal (13) recommended that cleansing of bottles should be carried out in three stages, washing, rinsing and finally sterilizing with 0·04 % chloramine which he considered preferable to sodium hypochlorite.

IV. *Reduction of contamination in sterilized milk*

(1) *At the farm*

Ridgway (6), testing raw milk from individual farms, found that samples with the lowest colony counts and satisfactory methylene blue tests had no better keeping quality after sterilization than poor-quality raw milk. In attempting to eradicate spores from the milk he concluded that instructing producers in efficient utensil cleansing alone exercised insufficient control.

It is clear that efforts should be made to reduce the sporeformer content of the raw milk, but attempts to control contamination by sporeformers at the farm will not be effective in improving the keeping quality of sterilized milk unless carried out in conjunction with control at the dairy.

(2) *Contamination of bottles*

It has been shown that cleansed bottles are a source of mesophilic contamination, and this should be eliminated if sterilized milk of good keeping quality is to be produced. Vindal (13) has suggested a provisional method for the chemical sterilization of bottles, but concluded that the only real solution lies in the construction of a machine specially designed for the work.

If the production of bacteriologically sterile milk by ultra high temperature processing (Cuttell (14)) develops, a sterile bottle will be essential, although there is also the problem of aseptic filling to be overcome.

(3) *Processing the milk*

Burton *et al.* (4) noted that the operation of milk-sterilizing pressure vessels could usually be considerably improved. They indicated the difficulties of producing bacteriologically sterile milk without undesirable brownness.

Careful operation of existing plant will reduce sporeformer contamination to a low level and improve the keeping quality of the sterilized milk.

5. SUMMARY

Samples of sterilized milk from fifteen dairies in the Midlands have been examined, and seasonal variations were detected in the incidence of mesophilic and thermophilic sporeformer contamination. Seasonal variation in the rate of spoilage at 30° C. was also apparent.

The lower incidence of contamination in summer was considered to be due to (*a*) less sporeformer contamination during milk production at the farm in the period when the cattle are not housed, and (*b*) increased processing times and temperatures in warm weather.

Cleansed milk bottles were examined and found to be an important source of contamination by mesophilic sporeformers, but the incidence of thermophiles of the type commonly found in sterilized milk was low.

Methods of reducing contamination in sterilized milk were discussed.

The authors gratefully acknowledge the co-operation of the managers of the dairies who supplied milk and bottles for examination. They wish also to thank staff of the Ministry of Agriculture, Fisheries and Food at Wolverhampton who have helped with the work, in particular Mr E. W. Malpass for his assistance in maintaining contact with the dairies and collecting samples. Mr W. E. Shotton has rendered invaluable assistance in the laboratory.

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637.128.61:576.8

640. SOME BACTERIOLOGICAL ASPECTS OF COMMERCIALLY STERILIZED MILK

II. TYPE OF SPOREFORMING BACTERIA ISOLATED

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(With 2 Figures)

1. INTRODUCTION

There have been few extensive surveys of the bacterial flora of commercial sterilized milk, but the recorded data indicate that it consists largely of strains of *Bacillus subtilis* and *B. lichen-formis*. Ridgway (1) classified 230 cultures isolated from commercial sterilized milk from one dairy, of which 89% were of the *B. subtilis/B. licheniformis* type, but these two species were not differentiated. Six other species were isolated, but only one strain belonged to the morphological group 2 of Smith, Gordon & Clark (2). Grinsted & Clegg (3) isolated forty-two cultures from commercial sterilized milk, of which some 79% were strains of *B. subtilis* and about 5% *B. licheniformis*. Only one other species was isolated, but this was probably due to the limited number of cultures isolated. Franklin, Williams & Clegg (4) isolated seven different species of aerobic mesophilic sporeforming bacteria from milk sterilized from ten sterilizing depots, and found that 44% of the strains isolated were *B. subtilis* and 26% were *B. licheniformis*. The number of strains examined was not recorded, and it is not known whether these results represent a typical cross-section of the bacterial flora. There was no apparent seasonal variation in the types isolated, of which a high percentage (13%) were unidentified.

Grinsted & Clegg (3) also examined thirty-six strains of aerobic thermophilic sporeforming bacteria from milk sterilized at one dairy and found all strains to be of a similar type, *B. calidolactis*, first described by Hussong & Hammer in 1928 (5).

The present study was undertaken to investigate the types of sporeformers present in commercially sterilized milk and the changes produced by them in milk, under conditions favourable for growth.

2. METHODS

Cultures were isolated and purified as described by Nichols & Candy (6) and identified by examination of the following characteristics, using the methods recommended by Smith *et al.* (2):

(*a*) Morphology.
(*b*) Growth on nutrient agar and glucose-nutrient agar.
(*c*) Ability to produce acetylmethylcarbinol.
(*d*) Starch hydrolysis. After incubation plates were flooded with dilute (Lugol's) iodine, which was found to give a clearer indication of hydrolysis than 95% alcohol.

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(*e*) Gelatin liquefaction.
(*f*) Growth in Koser's citrate medium.
(*g*) Growth in 5% NaCl broth.
(*h*) Anaerobic growth in glucose broth, using a vaspar seal.
(*i*) Growth at 65° C.
(*j*) Fermentation of glucose and lactose in a broth containing $(NH_4)_2HPO_4$ as the sole source of N.

In addition, Gibson & Abd- el-Malek's test (7), using a semi-solid glucose milk medium, was carried out to differentiate *B. subtilis* and *B. licheniformis*.

The reactions in litmus milk were also examined. These tests proved adequate for the identification of all mesophiles examined in the present work.

The egg-yolk reaction of McGaughey & Chu (8) was not used, the Voges-Proskauer test being relied on to distinguish *B. megaterium* from *B. cereus*.

Cultures were tested at the temperatures used for isolation, 30° C. for mesophiles and 63° C. for thermophiles.

3. RESULTS

I. *Mesophilic sporeformers*

(1) *Incidence*

Over a period of 3½ years from June 1952, 206 cultures of mesophilic sporeforming bacteria were isolated at random from samples of commercial sterilized milk showing spoilage when stored at 30° C. These cultures were identified as described above, and the results are shown in Table 1.

Table 1. *Sporeformers isolated*

	No. of strains	Percentage	
Group 1			
B. subtilis	126	61	74
B. licheniformis	26	13	
B. megaterium	12	6	
B. cereus	3	1	
B. coagulans	4	2	
Group 2			
B. macerans	6	3	
B. circulans	27	13	
B. brevis	2	1	
Total	206		

It will be seen that 74% of the strains isolated were of the *B. subtilis*/*B. licheniformis* type, which usually produces a soft coagulum with rapid digestion. 13% of strains were of the *B. circulans* type which usually produce slight acidity in milk without coagulation or digestion, although some strains may cause slow digestion (Table 2). The true incidence of *B. circulans* and other group 2 strains may be somewhat higher than the figures in Table 1 indicate. If *B. subtilis*, or other rapidly growing strains, were present in the milk incubated at 30° C. before plating, group 2 strains, which normally grow more slowly, would have been out-numbered and missed during isolation.

(2) *Seasonal variation*

The occurrence of the different strains of mesophilic sporeformers isolated was examined for indications of seasonal variation, particularly the incidence of *B. subtilis*/*B. licheniformis* strains in relation to other species. Results shown in Fig. 1 indicate a marked

seasonal trend in the incidence of *B. subtilis/B. licheniformis* types, the level being high in winter and lower in summer. 'Summer' has been regarded as the 6 months from 1 May to 31 October. It has been shown by Nichols & Candy (6) and by Ridgway (9), that in winter there is a greater incidence of contamination in commercial sterilized milk and

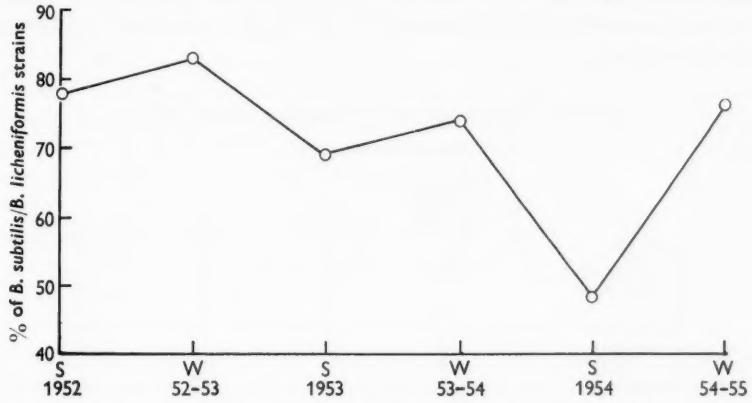

Fig. 1. Seasonal variations of *B. subtilis/B. licheniformis* strains expressed as a percentage of the total mesophiles isolated. S, summer months; W, winter months.

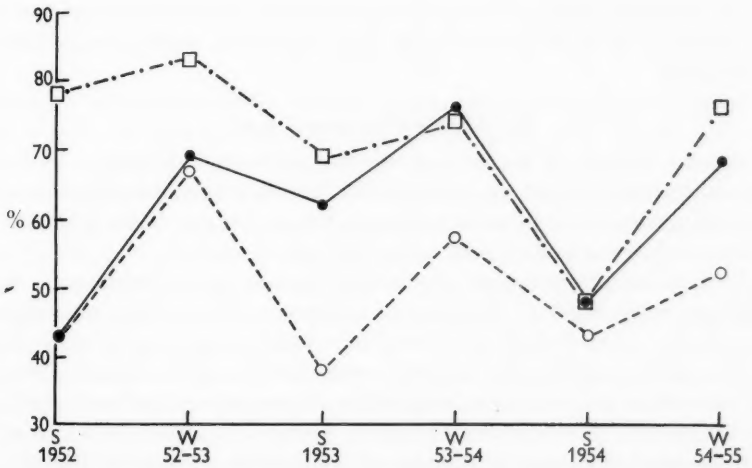

Fig. 2. Relationship between species and numbers of sporeformers, and spoilage rate at 30° C. ●——●, samples showing rapid spoilage (<5 days at 30° C.), see (6), Fig. 2; ○- - -○, samples showing high levels of contamination (level II), see (6), Fig. 3; □—•—□, *B. subtilis/B. licheniformis*, see Fig. 1.

more rapid spoilage in samples stored under conditions favourable to bacterial growth. It is suggested that the more rapid spoilage in winter is due to contamination by higher numbers of sporeformers, of which a greater proportion are the *B. subtilis/B. licheniformis* type, which usually produce rapid digestion (Fig. 2).

(3) *Action on litmus milk*

The reactions of the pure cultures in litmus milk incubated at 30° C. were studied, cultures being examined daily for 4 weeks. The results are presented in Table 2. Strains of *B. subtilis* usually produced rapid digestion (1–3 days at 30° C.) with alkaline or unchanged reaction, but 8 of the 126 cultures examined produced acid with slow digestion. Gibson (10) has also noted that some strains of *B. subtilis* produce a faint acidity and slow digestion in litmus milk.

Table 2. *Reaction of sporeformers in litmus milk*

	Soft clot and digestion			
Type	Unchanged or alkaline reaction	Acid reaction	Acid	Acid and gas
B. subtilis	118	8	0	0
B. licheniformis	25	1	0	0
B. megaterium	7	5	0	0
B. cereus	2	1	0	0
B. coagulans	1	1	2	0
B. macerans	0	0	0	6
B. circulans	5	9	13	0
B. brevis	2	0	0	0
Total	160	25	15	6
	185			

It will be seen from Table 2 that 185 (90%) of the 206 strains isolated produced a soft clot and digestion, the most common fault to be expected in sterilized milk held under warm conditions.

II. *Thermophilic sporeformers*

Thermophilic spoilage of commercial sterilized milk after delivery is rare in this country, although it can cause trouble if the milk has not been cooled sufficiently quickly after processing (Clegg (11)). In warmer climates, however, it may be an important factor in reducing the keeping quality, both before and after delivery.

Two hundred and fifteen strains were isolated from samples of commercial sterilized milk showing spoilage at 63° C. One hundred and seventy-three of these strains produced a firm acid clot in litmus milk, with reduction of the litmus usually within 24 hr. at 63° C. Three cultures produced a soft clot, with reduction of the litmus. Twenty-one cultures reduced the litmus without coagulation. Seven cultures produced slight acidity without coagulation. Eleven cultures produced gas and coagulated the litmus milk.

Differences were also noted in the type of thermophilic spoilage by Burton, Akam, Thiel, Grinsted & Clegg (12), but the thermophiles isolated by Grinsted & Clegg (3) produced only one type of reaction, a firm acid clot, in litmus milk.

A preliminary examination of twenty-four cultures, excluding those producing gas, was begun. All were of the group 2 morphological type and grew at 65° C., which suggested that they were strains of *B. stearothermophilus*, according to the classification of Smith *et al.* (2). However, only two of these cultures liquefied gelatin, the remaining twenty-two being unlike *B. stearothermophilus* in this respect. The two cultures capable of liquefying gelatin produced acetylmethylcarbinol which could be detected by the

methods used by Smith *et al.* (2), and were again unlike *B. stearothermophilus* in this respect. Thus none of the twenty-four cultures capable of growth at 65° C. strictly resembled *B. stearothermophilus*. These cultures also differed from *B. calidolactis* in several major characteristics, two strains liquefying gelatin and producing acetylmethylcarbinol. All strains belonged to the morphological group 2.

Production of gas at 63° C. It was noticed occasionally that samples incubated at 63° C. showed the production of gas. Gas was produced during the first day's incubation and could be detected before coagulation of the milk. After coagulation the firm curd was channelled because of gas production. This type of spoilage could only be repeated on subculture by using a very large inoculum (10%) of a young active culture. The causative organism was found to be *Clostridium thermosaccharolyticum* (McClung (13)), which can grow in media of low redox potential or under anaerobic conditions.

The incidence of *Cl. thermosaccharolyticum* was investigated by adding gentian violet to bottles of sterilized milk in a concentration of 1:100,000 and incubating at 63° C. to reduce spoilage due to aerobic thermophiles and permit the growth of any anaerobic thermophiles present (Cameron (14)).

Of the forty-four bottles examined in this way, thirteen (29·5%) have shown the presence of *Cl. thermosaccharolyticum*. It is probable that the concentration of the dye added could be increased, as at present spoilage due to thermophiles other than *Cl. thermosaccharolyticum* still occurs to some extent.

In the absence of the inhibitory dye, the presence of *Cl. thermosaccharolyticum* is usually masked by the growth of aerobic thermophiles.

4. DISCUSSION

Contamination of sterilized milk by *Bacillus subtilis* and by thermophilic sporeformers is common. There is general agreement on the high incidence of *B. subtilis*, but there is some confusion on the species of thermophiles present.

Grinsted & Clegg (3) conclude that *B. calidolactis* is the only thermophile present in sterilized milk, but this species, which can grow at 65° C., is not recognized by Smith *et al.* (2), who maintain that *B. stearothermophilus* is the only aerobic sporeformer capable of growth at that temperature. Smith *et al.* (2) examined five of the cultures of *B. calidolactis* originally isolated by Hussong & Hammer (5) and identified three as *B. coagulans* and the remainder as *B. stearothermophilus*.

The thermophiles described by Grinsted & Clegg (3) as *B. calidolactis* differ from the original description of this organism (5) in showing no stimulation of growth on addition of glucose to the medium.

Some important characteristics of aerobic sporeformers capable of growth at 60° C. are compared in Table 3. It will be noted that the thermophiles studied in the preliminary examination could not be strictly identified with any of the known thermophilic strains. Marsh & Larsen (15), examining thermophilic sporeformers from hot springs, also encountered strains capable of growth at 65° C. which differed significantly from *B. stearothermophilus*.

The classification of aerobic thermophilic sporeformers appears to be more complex than earlier workers have suggested, and it will clearly be necessary to examine this problem in greater detail.

The heat resistance of *B. subtilis* and the thermophilic sporeformers present in sterilized milk is high and under favourable conditions rapid spoilage is produced. Elimination of these organisms by increasing treatment times and temperatures with the types of plant in use is not possible without browning the milk and causing a deterioration in flavour. By using an ultra high temperature heating system (Cuttell(16)) contamination could be reduced to a very low level without any adverse effect on the milk. It is unlikely that this method of processing will be fully effective unless a system of aseptic filling into sterile bottles can be developed, as both the filler(12) and the bottles(6) are important sources of sporeformer contamination. In this country it seems that little general improvement in keeping quality would result from processing at ultra high temperatures. In warmer climates, however, the growth of thermophilic sporeformers during storage after processing may be an important factor in reducing the keeping quality, and ultra high temperature treatment might remedy this.

Table 3. *Characteristics of aerobic sporeformers capable of growth at* 60° C.

Type	Growth at 65°	V–P*	Liquefaction of gelatin	Litmus milk reduced acid clot	Morphological group
B. coagulans	–	Usually +	–	+	Intermediate 1/2
B. calidolactis	+	–	–	+	Intermediate 1/2
B. stearothermophilus	+	–	+	+	2
B1/3T† (2 strains)	+	+	+	+	2
B4/1T† (22 strains)	+	–	–	+	2

* Method of Smith *et al.* (2) used.
† Isolated from commercial sterilized milk by the authors.

5. SUMMARY

Cultures of sporeforming bacteria isolated from commercially sterilized milk were examined and seasonal variations in the incidence of *B. subtilis*/*B. licheniformis* strains were apparent which may account for the increased rate of spoilage at 30° C. during the winter.

B. subtilis was the most common mesophilic sporeformer isolated, and under favourable conditions it produces rapid digestion of milk.

Thermophilic sporeformers are frequently present, and usually produce a firm acid clot in milk held at temperatures favourable for their growth. The classification of the thermophiles isolated is uncertain and a review of the classification of the aerobic thermophilic sporeformers in general is required.

Cl. thermosaccharolyticum, a gas-producing thermophile, was isolated from sterilized milk.

The authors are grateful to Mr W. E. Shotton for technical assistance throughout the work.

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641. PENICILLIN IN MILK

I. THE RAPID ROUTINE ASSAY OF LOW CONCENTRATIONS OF PENICILLIN IN MILK

A joint survey by The National Institute for Research in Dairying and
The Bacteriologists of the National Agricultural Advisory Service
co-ordinated by

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When it became apparent that the satisfactory manufacture of cheese was threatened by the contamination of milk with penicillin, which is now injected into cows for the treatment of mastitis, suggestions for a rapid method of recognizing the contamination were published (1). We hoped that creameries would thus be enabled to reject milk containing penicillin before it reached the vat. Although this end was not realized, the techniques suggested have been used in a survey (2), and we propose to describe the results of the wide experience which has thus been gained. Eight laboratories of the National Agricultural Advisory Service have co-operated in the survey. More than 5000 samples of milk have been examined. There are, of course, numerous methods of estimating the penicillin which may be present in milk; see, for example, Neal & Calbert (4), Mattick, Anderson & Wildasin (5) and the review by Overby (6). The method to be described here was chosen for its simplicity, sensitivity and rapidity.

METHOD

The concentration of penicillin was determined by mixing the milk sample with an actively growing culture of *Streptococcus thermophilus* and comparing the acidity produced after a short incubation with the acidity in control mixtures containing known concentrations of penicillin. *Str. thermophilus* was chosen because it is very sensitive to penicillin (3). Penicillinase was used to distinguish between penicillin and other inhibitors of bacteria.

Culture

This was a strain of *Str. thermophilus* (N.I.R.D. no. 7) chosen for its rapidity of growth and high sensitivity to penicillin. Stocks were kept in yeast glucose agar stabs, bullock's heart medium or litmus milk and chalk. The culture for daily use was refrigerated over the week-ends, but at other times it was grown during the day in yeastrel glucose broth at 45° C. and subcultured into litmus milk for growth overnight at 37° C. This appeared to maintain a vigorous culture well adapted to milk. Cultures freshly taken from stock were subjected to this routine for at least a week before use. Two laboratories used 37° for incubation both during the day and over the week-end when the culture was kept in litmus milk and chalk.

Actively growing culture

The preparation of this was begun by inoculating 10 ml. of overnight culture in litmus milk into 100 ml. of a mixture consisting of 25% laboratory-pasteurized milk and 75% sterile yeastrel glucose broth and containing 0·01% bromo-cresol purple. The culture was maintained at 45° C. After this point no asepsis beyond the use of sterile broth and pasteurized milk was practised. The freshly prepared culture had a blue or bluish grey colour. As growth proceeded acid was formed and the indicator changed gradually through grey and green to yellow. Later still the casein of the milk coagulated. Shortly after this the rate of growth of the streptococcus diminished. Since cultures often take a considerable time to regain a high rate of growth once they have passed out of the logarithmic phase care was taken to avoid keeping the culture in the clotted condition for any appreciable length of time, and in fact it was usually diluted with fresh '25/75' medium before it had reached this condition. It was found most convenient to make the dilutions every hour according to the instructions in Table 1. In this way a considerable volume of culture in its logarithmic phase of growth, suitable for use in the assay, could be built up within 2–3 hr., and could be maintained throughout the day for use at short notice.

Table 1. *Hourly dilution procedure for maintaining the test culture in an actively growing condition*

Colour and condition of culture	Volume of culture to be added to 50 ml. of fresh medium at 45° (ml.)
Green-greenish yellow	25
Yellow or dirty yellow, not clotted	15
Yellow and clotted	10

Penicillin standard

Crystalline procaine penicillin was used as a working standard and was compared occasionally with standard sodium penicillin.* Care was taken to keep the stock of standard powder dry during storage and when removing samples. Phials taken from the refrigerator were not opened until they had reached room temperature so that atmospheric water would not condense on the powder. Standard solutions were prepared by dissolving procaine penicillin in 0·05 M phosphate buffer at pH 6·8–7·0 to give a concentration of 8 mg./100 ml. The solution, which was, of course, kept in the refrigerator, was used for up to 2 days after its preparation, but no longer.

Penicillinase

'Wellcome' penicillinase was used at a final concentration of 0·5% in the milk to be tested. This was later increased to 1·0% (see below).

Technique

All the glass-ware was particularly clean and free from detergents, but it was not sterile.

A control series of test-tubes containing known amounts of penicillin was prepared by

* Kindly supplied by Dr Muggleton of Glaxo Laboratories Ltd.

diluting the standard solution 400-fold (giving 0·2 μg./ml.) in distilled water and measuring 1·0, 0·5, 0·25, 0·12, 0·06 and 0·03 ml. into a series of test-tubes. To each tube was added 2·5 ml. of pasteurized penicillin-free milk containing 0·05% bromo-cresol purple.

Each unknown sample was prepared by adding bromo-cresol purple to a concentration of 0·05%. Two aliquots of 2·5 ml. were measured out and to one of them was added 0·5% of penicillinase (e.g. 0·25 ml. of 5% solution). The unknown samples were also diluted tenfold and 100-fold in penicillin-free pasteurized milk and samples of these dilutions prepared in the same way. To all these samples and to the control series 2·5 ml. of a dilution of the log phase culture was added.

The dilution normally used was 2% of the culture in the special '25/75' mixture used for maintaining it. It was found preferable to use the culture at the grey stage. The green culture was not vigorous enough and if it had become yellow and clotted it was too near the end of its logarithmic phase, and it was therefore necessary to dilute it and allow growth to proceed to the grey stage before the culture could be used.

After 2½ hr. incubation of the tests and controls in a stirred water-bath at 45° the colours of the indicator in the unknown samples were compared with those in the control series, and an estimate of the penicillin content was made. When there was inhibition of the test organism in samples containing penicillinase the samples were noted as containing inhibitors other than penicillin.

The first tube of the control series contained 0·2 μg. procaine penicillin, i.e. approximately 0·2 i.u. If the volume of the penicillin solution be neglected the series thus corresponds to samples of milk containing 0·08 (0·2/2·5), 0·04, etc., i.u./ml., and these figures have been retained as nominal values which approximate more and more closely to the true values as the series is descended. The true value for the first tube is 0·067 i.u./ml., for we have 0·2 i.u. in 6 ml. of mixture in the standard tube, i.e. 0·033 i.u./ml., but an unknown sample corresponding exactly to this will contain half milk and half culture, so that the concentration in the milk was 0·0666, or say 0·067 i.u./ml. before dilution. Similarly, the second and third tubes correspond to milk containing 0·036 and 0·019 i.u./ml.

Two of the laboratories modified their procedure to give controls containing 0·1, 0·05, 0·025, etc., i.u./ml.

When time was short a modification of the technique was used. This consisted in the use of the grey culture itself in place of the 2% dilution, all other conditions remaining the same. The resulting 50-fold increase in inoculum diminished the sensitivity of the assay towards low concentrations of penicillin, but not so much that a different series of standards was needed. The advantages of this technique were that readings could usefully be taken after 30 min. incubation, and that the effect of other micro-organisms was made very small by the overwhelming preponderance of the test organism.

A number of minor variations used in different laboratories have not been included in this description.

RESULTS

The sensitivity of the test depended on the conditions, especially on the size of the inoculum and the time of incubation as was also observed by Neal & Calbert (3). It was easy to detect 0·01 i.u./ml. and frequently 0·005 i.u./ml. could be measured. When the rapid technique was used the minimum amount detectable in half an hour varied from 0·06 to 0·015 i.u./ml. (1).

Five of the eight laboratories used the assay without remarking on difficulties or sources of error. The other three observed that acid was produced at different rates in different milks; for example, the standard milk to which known amounts of penicillin were to be added did not always support growth at the same rate as did various other samples known to be free from penicillin. Thus the results, it was felt, would be in error. The effect is exemplified in the following experiment in which samples were chosen to demonstrate a large error.

The rates of growth of the test organism in seven single farm milk samples, free from penicillin, were compared, and the two milks showing the maximum difference were selected. Identical quantities of penicillin were added to aliquots of each milk, and after inoculation and incubation in the usual way the apparent levels in the first milk were determined, using the second one as a standard. The results are shown in Table 2, from which it is clear that even in this special case, the errors are serious only below 0·04 μg./ml. This is in agreement with another laboratory which advocated reserve in interpreting results below 0·04 μg./ml.

Table 2. *The effect of differences in milk on the apparent penicillin concentration*

True value (i.u./ml.)	Observed value (i.u./ml.)
0·08	0·08
0·04	Slightly more than 0·04
0·02	Slightly less than 0·04
0·01	More than 0·02
0·00	Slightly more than 0·02

Experiences of this kind led some workers to search for a better standard milk. One of them was happy with sterilized separated milk, of which a large quantity was prepared (by steaming on 3 successive days) and stored in the laboratory. Others found growth in sterilized milk too fast in comparison with that in the samples. Another worker preferred raw milk from a single selected cow. Several laboratories experimented with reconstituted skim or whole milk powder. The opinions were not all favourable, probably because the response of the test organism depends on the milk powder, but the experiences indicated that it would probably not be difficult to discover, by examining a number of samples, a suitable powder which would form a readily reproducible standard substrate for use in different places at different times. Reconstituted milk was used for a significant proportion of the survey samples in some laboratories.

Failure of the control tubes to form a regularly graded series, particularly at low concentrations of penicillin, occurred on some occasions and no one was able to trace the cause. It seems likely that when the concentration of penicillin is very low minor variations in conditions or technique affect the growth of the test organism, and that more rigid control is necessary if very low concentrations are to be measured with any degree of accuracy.

The test organism grows more rapidly in milk that has been boiled or autoclaved than in raw milk. It also grows very rapidly in some samples of milk reconstituted from powder, the extent of the stimulation probably depending on the degree of heating the milk powder has received during manufacture. Although the test organism is stimulated when growing in autoclaved milk, it is possible that more severe heating would diminish the stimulatory properties and that storing the autoclaved milk might have a similar

effect (see, for example, Auclair(7)). These factors may help to explain why different laboratories found different reactions of the organism towards autoclaved milk. (For a review concerning inhibitory and stimulatory properties of milk see Berridge(8).)

The variations and sources of error which have just been discussed were diminished by the use of penicillinase although not all the workers were satisfied with it. One found 0·5% occasionally inhibitory. Another found 0·5% insufficient if the penicillin concentration was high, and recommended 1·0% or a preliminary contact time of 1 hr. if penicillin concentrations as high as 0·5 i.u./ml. were expected. If, however, such a strong inhibition is observed the assay would depend on one of the diluted samples in which the penicillinase would be effective.

Slow growth due to causes other than penicillin could be recognized by the use of penicillinase, but when the portion of milk so treated showed much better growth than the portion not treated, and also better growth than the standard containing no penicillin, it was impossible to say how much penicillin was present. This is again the result of the different inhibitory properties of different milks. Table 2 suggests that it is important only when the penicillin concentration is low. Especially is this so because marked slowness in the standard milk would lead to its being discarded for a better one.

It thus appears that if a worker were using, as a standard, a milk containing *less* than the average concentration of inhibitory substances, he would report a falsely high proportion of positive but low values for inhibitor content, and vice versa. This may, in part, account for the observed variation between the results from different laboratories, but since the probability of all laboratories choosing standard milk of similar properties is small, there is reason to believe that the overall average for the whole survey can be accepted.

It was felt that the samples should preferably not be heated, and most laboratories examined them on the day of collection without pasteurizing them. At one place this was often not possible because of the long distances between the creameries and the laboratory, and because samples taken from the cheese vats always contained starter. This laboratory pasteurized some of the milk samples at 62·5–65° C. for 30 min. and stored them overnight in the refrigerator. Several series of tests were conducted during the period of the survey, and it was shown that the treatment had no observable effect on the penicillin content of the samples. It has also been shown that momentary heating to 94° C., which was achieved by 2 min. immersion in boiling water, has no effect on penicillin concentration in milk. It is possible that such a treatment would reduce the differences in inhibitory properties of milk, since some at least of the inhibitors are thermolabile.

CONCLUSIONS

The experience summarized in this paper shows that it is practicable to carry out dilution assays of low concentrations of penicillin in milk by the use of a culture which is maintained in its logarithmic phase of growth.

By the use of a 'massive' inoculum of actively growing bacteria the technique combines the advantages of ease and rapidity of manipulation (because asepsis is not required) with those of speed in the production of results.

The results of some experiments suggest that in future reconstituted milk from a selected powder would form a better diluent for the control series than fresh milk.

Even so, it will remain difficult if not impossible to unravel completely the interplay of inhibitory and stimulatory factors in milk samples when the concentration of antibiotic is very low. The assay of many samples on the day of collection is often inconvenient, because several hours may elapse before the assays are finished. There are two ways of avoiding this. One is to use the large inoculum and to take readings after half an hour, neglecting thereby very low concentrations of penicillin; the other is to pasteurize samples and store them in the refrigerator. We now know that either of these methods can safely be used.

Experiments to be reported later suggest that these low levels are not deleterious in cheese-making, but the measurement of them, even if only approximate, can provide a useful warning that dangerous levels are beginning to be approached. One might, for example, foresee that a doubling of the sizes of the doses of penicillin now administered or of the number of cows treated would lead to serious losses in the cheese-making industry.

SUMMARY

Experience in the use of a rapidly growing culture of *Streptococcus thermophilus* for the assay of traces of penicillin in milk is described.

We wish to acknowledge with thanks the part played by Dr A. T. R. Mattick in instituting the work recorded in this and the following paper and in promoting its completion. Our best thanks are also due to the Provincial Advisory Bacteriologists, namely, Mr H. Barkworth, Mr W. A. Cuthbert, Mr J. W. Egdell, Mrs P. M. Hobson, Dr A. A. Nichols, Mr C. A. Scarlett and Mr S. B. Thomas, for their encouragement and help. The following members of the National Agricultural Advisory Service were responsible for the actual laboratory work: Miss E. M. Allen, Miss M. Bateson, Dr E. Billing, Mr F. W. Catton, Miss M. Edge, Miss M. A. Edwards, Miss K. L. Lomax and Mr J. J. Panes. Finally we wish to thank Mr G. C. Cheeseman for assaying the procaine penicillin samples used as standards.

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642. PENICILLIN IN MILK

II. THE INCIDENCE OF PENICILLIN

A joint survey by The National Institute for Research in Dairying and
The Bacteriologists of the National Agricultural Advisory Service
co-ordinated by

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It is impossible to make normal hard-pressed cheese with milk containing penicillin in amounts sufficient to prevent the growth of starter bacteria. Thomas, Panes & Lewis (1), who have reviewed the previous literature, have demonstrated that even when low concentrations of penicillin are present so that acidification by the starter bacteria is only diminished and not prevented, the cheese is likely to be severely down-graded.

It is well known that penicillin infused into cows' udders is excreted with the milk during several subsequent milkings, and in spite of agreements to the contrary a considerable quantity of milk containing penicillin reaches the market. In the absence of definite information penicillin in milk may be considered a real obstacle to the manufacture of good cheese, or a mere bogy and convenient scapegoat. The results which follow, together with those of the third paper of this series (2) should assist in deciding which, in fact, it is. A survey with this end in view was recorded by Storrs & Hiett-Brown (3), while the present work was in progress. In this work we have cast the net wider and fished later; it is therefore not surprising that our catch is a little different, though not seriously divergent.

PLAN OF WORK

Laboratories of the National Agricultural Advisory Service situated in Cambridge, Starcross, Leeds, Bristol, Wolverhampton, Derby, Bangor and Aberystwyth took part in the survey. To secure uniformity of techniques the workers concerned met together from time to time to discuss methods and progress. Samples were collected as the urgency of other work, the geographical situations of the laboratories and other circumstances permitted. For this reason different regions have contributed different proportions of the total results. While interest was focused chiefly on milk as found in the creamery vat, other types of samples were not despised. Consequently, we have also a number of results for mixed milk of single farms and some for single churn samples.

All the workers were conscious of the difficulties of securing representative samples from bulks of over 300 gal., and each one took the action appropriate under the circumstances. For example, when cheese vats could be stirred the difficulty was diminished; at other times several samples were taken from different places in one vat and the samples were mixed. Other samples were taken from tipping tanks. Most of the cheese vats were sampled when they were only half full, and account of this has been taken in the record of bulks sampled in Table 2.

METHODS

The method of assay has been described in Part I (4). The use of penicillinase permitted inhibitory substances other than penicillin to be recognized, and the opportunity was taken to record the occurrence and the equivalent concentration of these substances.

Standard penicillin was distributed to each laboratory in the form of several phials of procaine penicillin taken from a single batch. In September 1954 samples of the standard were collected from the laboratories and assayed at the Institute in duplicate on two occasions according to the method of Berridge & Barrett (5); most of the assays gave two readings so that eight readings were available for each sample. All the samples gave average readings between 900 and 1100 except one which was 1120 i.u./mg. At the end of the survey samples of standard were again assayed in a similar way, and the results all fell between 700 and 850 i.u./mg. These results were obtained by comparison with a sodium penicillin standard.* Since it was proposed to measure the penicillin concentrations to the nearest power of 2, no correction has been made for these deviations of the procaine standard from the assumed value of 1000 units/mg.

In spite of the contact between workers differences in technique were inevitable owing to the different circumstances. Most of these differences arose in the treatment of samples, choice of control milk, etc., and they have been discussed in Part I (4). At the levels of accuracy attempted they are probably not important.

RESULTS

The results of the survey are presented in Tables 1–5 and in the notes appended to them. A few additional notes concerning separate districts are included.

(1) *Seasonal trend*

In three districts it was possible to examine the results for seasonal trends, particularly with single farm samples. The results are shown in the following table and they do not appear to indicate any seasonal trend. No result is from less than ten samples.

Table 1

Month	Percentage of single farm samples containing 0·01 i.u./ml. or more		
	Wolverhampton	Bangor	Aberystwyth
April	—	—	8
May	—	—	10
June	—	—	12
July	—	16	8
August	—	7	5
September	—	3	19
October	—	7	18
November	—	2	19
December	8	4	—
January	13	5	15
February	8	3	—
March	11	7	—
April	6	4	—
May	8	4	—
June	9	3	—
July	11	1	10
August	—	3	—
September	—	2	5

* This was kindly supplied by Dr P. W. Muggleton, of Glaxo Laboratories Ltd., Greenford, Middlesex.

(2) *Incidence of penicillin in bulk milk*

Samples from cheese vats, weigh tanks and transport tankers collected during periods of 5–17 months between April 1954 and September 1955 gave the results reported in Table 2.

Table 2. *Summary of results for bulk milk*

District	Size of bulk (gal.)	No. of samples	Proportion containing (*a*) 0·01 i.u./ml. or more (%)	Proportion containing (*b*) 0·04* i.u./ml. or more (%)	Penicillin content of samples ≥ 0·04* (i.u./ml.)
Cambridge	300–500	66	4·9	3·0	0·06, 0·30
Starcross	700–750	45	47	< 2·2 (i.e. not one sample)	—
Leeds	300–500	170	8·8	0·6	0·05
Bristol	300–3000	328	4·9	2·4	7 at 0·08
Wolverhampton	150–250	36	25	2·8	0·05
Derby	300–600	95	15·6	5·2	0·08, 0·10, 0·20, 0·80
Bangor	300–1000	364	11	0·8	0·05 and 2 at 0·10
Aberystwyth	350	327	7·4	4·0	3 at 0·01 4 at 0·05 1 at 0·15† 3 at 0·30† 2 at 0·4–0·6†

* Nominally 0·04, actually 0·036, as explained in Part I. † See note under 'Aberystwyth'.

(3) *Incidence of penicillin in the milk of single farms*

The frequency with which the two designated levels were exceeded is shown in Table 3. Of more interest is the total output of penicillin by each farm. It was possible to calculate this in those cases where records of milk volume had been made. These results are shown in Table 4. Further details may be found in the notes relating to each district.

Table 3. *Summary of results for single producers*

District	No. of samples	Proportion containing (*a*) 0·01 i.u./ml. or more (%)	Proportion containing (*b*) 0·04* i.u./ml. or more (%)
Cambridge	124	9·7	4·8
Starcross	193	18·5	4·1
Leeds	72	6·9	1·4
Bristol	654	7·5	4·7
Wolverhampton	944	9·4	3·9
Bangor	1300	4·2	2·4
Aberystwyth	626	12·6	4·8

* Nominally 0·04, actually 0·036, as explained in Part I.

Table 4. *Penicillin levels and frequency among samples from single farms*

Penicillin levels (Gal. × i.u./ml.)	Number of samples: Bristol	Cambridge	Starcross
Zero	607	114	178
0 – 2·5	21	7	12
2·5– 5·0	15	0	2
5 –10	4	2	1
10 –15	3	0	0
15 –20	1	0	0
20 –25	2	1	0
45 –50	1	0	0

(4) *Single churn samples*

Less effort was expended on these, but 302 samples were examined in Wolverhampton and 5·3% of them contained more than 0·01 i.u./ml. In the Cambridge area 273 single churn samples each from 10 gal. and twenty-three samples from 4 to 12 gal. gave sixteen positives (5·4%). Of these, two contained >0·8 i.u./ml., one contained 0·4 and one 0·15. The levels in the rest were <0·08 i.u./ml. In the Leeds area no positive sample was found in the fifty-six tested.

(5) *Occurrence of inhibitors other than penicillin*

In some districts these inhibitors were detected frequently, but always at low levels. The maximum recorded was equivalent to 0·04 i.u. penicillin/ml. The percentage of samples containing inhibitors equivalent to 0·01 i.u. penicillin/ml. or more, are given in Table 5. In Bristol the proportion of these inhibitors recorded was vanishingly small, probably because most of the samples were pasteurized before testing. The Leeds laboratory found that all the inhibitors which they observed were heat labile. It seems possible that we are here dealing mostly with relatively high concentrations of an inhibitor similar to lactenin which, as Auclair & Hirsch have shown (6), is considerably diminished by heating. The effect of legal pasteurization has also been studied by Auclair (7).

Table 5

	Percentage of samples showing inhibition not due to penicillin	
District	Samples of bulk milk (%)	Mixed milk of individual farms (%)
Starcross	0	4·7
Leeds	20·5	30·6
Wolverhampton	14	24·5
Bangor	5·5	15·2
Aberystwyth	8·6	21·1

Additional notes on the results from each area

Cambridge

Among eight bulk samples taken from 100–250 gal. were four positive ones, three containing 0·06 i.u./ml. and one 0·08. The lower bulk volumes and the very high incidence of penicillin suggest that these may not have been random samples.

The 124 farm samples recorded in Table 3 included sixteen which, from their volume and source, were presumably from single producers, although they were not definitely recorded as such.

The total volume of all the single producers' milk sampled was 6981 gal. The total output of penicillin during the whole period was 44·2 gal. × i.u./ml., and the highest single output was 23 gal. × i.u./ml. (115 gal.; 0·2 i.u./ml.). The dilution of this to a mere 250 gal. with penicillin-free milk would have brought the level below the really dangerous threshold of 0·1 i.u./ml.

Four samples from 1500 gal. tankers were found to be negative.

Twenty-four samples have been omitted from the above records, since they do not fall into any of the chosen categories.

Starcross

There were 109 farm samples with recorded volumes giving a total of 3553 gal. The highest penicillin output from one farm was only 5·6 gal. × units/ml. (7 gal.; 0·8 i.u./ml.). This region is notable for the absence of high levels of penicillin.

Bristol

Of the seven samples reported to contain 0·08 i.u./ml., four came from bulks of 300–415 gal., one from 750, one from 1000 and one from 3000 gal.

Among the results for single producers given in Table 3 are a number obtained from a survey of the milk of three groups of producers in different districts around Bristol. Samples of milk were taken at the buyer's premises at approximately 3 to 4-week intervals. Each sample was representative of all the p.m. and a.m. milk.

In group 1, twenty-one supplies were sampled on fourteen occasions (occasionally a supply was missed), over a period of a year. Of the 288 samples taken thirty-four samples (11·8%) contained 0·01 i.u./ml. or more penicillin. Nine supplies had positive samples on at least two occasions, and nine samples out of fourteen from one producer were positive. Only eleven out of the twenty-one producers had penicillin-free samples during the whole period.

In group 2, twenty supplies were sampled five times from January to July. Out of 100 samples only two contained 0·01 i.u./ml. or more.

In group 3, ten supplies were sampled five times from January to July, and seven samples (14%) out of fifty contained 0·01 i.u./ml. or more.

From these surveys it is seen that penicillin was present in more than 10% of the samples for two groups of farms but the incidence was lower in the other group.

Aberystwyth

The six high results marked in Table 2(†) were obtained from six cheese vat samples taken on the same day. The cheesemaker was able to produce marketable cheese from four of these vats. It should be borne in mind that only one 6 oz. sample was taken from each 700 gal. vat and it is possible that such a sample may not give an accurate representation of the true penicillin content of such a large volume of milk.

DISCUSSION

When the survey was planned the possibility of low concentrations of penicillin interfering with the ripening of cheese was borne in mind. For this reason it was felt desirable to determine the lowest concentration which could reasonably be observed. This seemed to be about 0·01 i.u./ml. At this level the test as used was felt by some of the workers not to be very reliable. This point has been discussed in Part I(4). It was also found that these levels occurred relatively often. For this reason we have also recorded the frequency of occurrence of levels equal to or above 0·04 i.u./ml. Recent cheese-making experiments at this Institute have suggested that the low levels of penicillin which do not influence the activity of the starters have little, if any, effect upon the subsequent ripening of cheese. Some starters form acid at the normal rate even in milk containing 0·1 i.u. of penicillin/ml. (See Part III.)

Thomas, Panes & Lewis(1) showed that it was possible to make cheese, although grade III, from milk with 0·16–0·2 i.u./ml. Probably the grade could be improved in normal practice by judicious curd mixing. The results of Table 2 show that during the period of the survey the concentration of penicillin in bulk milk rarely exceeded 0·1 i.u./ml. and thus that slowness in cheese-making or poor quality of cheese could seldom be attributed to the presence of penicillin.

Owing to the mixing of milk from different sources it is obvious that the incidence of lower concentrations of penicillin will be more frequent than that of the higher concentrations. This is, in fact, borne out by the results of Table 2.

Although 0·04 i.u./ml. may not be deleterious, its measurement provides a useful preliminary indicator or warning. Thus the results for this level show that if the concentration of penicillin were increased between 2 and 3 times by increased dosage or increased frequency of treatment, up to 5% of bulk samples would contain quantities of penicillin, likely to inhibit the growth of starter cultures and therefore to cause difficulty in cheese-making.

In some districts it was possible to record the grading of cheese prepared from the vats which had been sampled. Inspection of these results showed quite clearly that there was no correlation between the grade achieved and the presence or absence of penicillin in the milk. For example, batches of cheese all of the same grade originated partly from milk containing penicillin and partly from normal milk. This is in agreement with the view expressed above, that the low concentrations of penicillin which have been observed are insufficient to influence the ripening of cheese. It is important, however, to emphasize that under the present conditions an increase of two- to threefold in the quantity of penicillin in use for the treatment of dairy cows might well plunge the cheese-making industry into severe difficulty.

SUMMARY

An examination of over 5000 milk samples has shown that penicillin is frequently present. Its concentration, however, is usually below the level which is believed to be necessary to cause significant interference in cheese-making, but a significant proportion of the samples contained more than a third of this quantity. This suggests that conditions under which difficulty would arise are not remote.

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637·333·13

643. PENICILLIN IN MILK

III. THE EFFECT OF LOW CONCENTRATIONS OF PENICILLIN ON THE RATE OF ACID PRODUCTION BY STARTER CULTURES

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(With 12 Figures)

In order that the results recorded in Part II of this series (1) may have meaning, it is necessary to know the effect of different low concentrations of penicillin in cheese-making. Preliminary experiments suggest that this is mainly the result of their effect on the cultures used as starters.

There are in the literature a good many statements about the sensitivity of important dairying bacteria to penicillin, as may be gathered from the review by Overby (1) and, in spite of occasional deviations, there seems to be general agreement that at least 0·1 i.u./ml. is necessary to cause slight inhibition of *Streptococcus lactis*. Mattick, Anderson & Wilderin, for example, report the reduction of titratable acidity from 0·1 in the control to 0·09 in milk containing 0·1 i.u./ml. together with other results in the same vein (2). Neal & Calbert, on the other hand, state that commercial lactic starter was inhibited by 0·3 unit (3). Stoltz & Hankinson (4), using Hansen's lactic ferment, found an inhibition of acid production at 0·1 unit/ml. This inhibition was only slight, although the bacterial counts diminished considerably instead of increasing in the normal way. The effect of 1·0 unit/ml. was of course devastating and that of 0·01 insignificant.

Several years ago Hunter (5) measured the effects of different concentrations of penicillin on the rates of acid production of cultures of *Str. lactis* and *Str. cremoris* which were in use as starters in New Zealand. He found a definite slowing down of *Str. cremoris* at 0·05 i.u./ml., but only slight effects on cultures of *Str. lactis* at concentrations of 0·10 i.u/ml.

While it would be fairly safe to assume from Hunter's thorough work that 0·05–0·1 i.u./ml. is the maximum permissible level of penicillin in milk for cheese-making, it is clearly advisable to test the actual strains which are now in use in this country. Accordingly, a number of cultures of *Str. lactis* and *Str. cremoris* selected for their rapid acid production and suitability for cheese-making were obtained from the National Collection of Dairy Organisms maintained at the Institute. Since, in the making of cheese on a large scale, timing is so important the rates of acid production of these cultures in milk with various concentrations of penicillin were measured.

METHODS

A solution of standardized procaine penicillin was prepared containing 1768 i.u. in 250 ml. of M/100 phosphate buffer at pH 7·2. Serial dilutions were prepared from this in the same buffer using the syringe technique of Berridge & Barrett (6). The syringe contained 5 ml. and delivered 2·5 ml. This volume, which contained 17·68 units of penicillin, was therefore delivered into the first of a series of boiling tubes of about 60 ml. capacity each. The

now half-empty syringe was refilled with fresh buffer, diluting the contents twofold, and 2·5 ml. was then delivered into the second tube. This therefore contained 8·84 units of penicillin. Seven boiling tubes prepared in this way constituted a set of serial dilutions. Two such sets were prepared for each culture to be tested, and an empty tube for zero penicillin was added to each set. Thus the experiment was done in duplicate.

When all the sets of boiling tubes were ready the cultures were prepared by diluting 7 ml. of the overnight culture in litmus milk to 700 ml. in raw milk. Fresh morning's milk from the Institute herd was used. This was bulked and carefully mixed to avoid variations in inhibitory properties which occur between the milks of different cows. The small volume of clotted overnight culture was thoroughly liquefied by using a pipette with a narrow orifice for the measurement. In addition, the precaution was taken of centrifuging the diluted culture to remove clots or large clumps of bacteria. This precaution was not, however, taken in a second experiment, and it may be for this reason that the duplicates show slightly poorer agreement. The 1% culture so prepared was added to the duplicate sets of boiling tubes at the rate of 43·6 ml. per tube using a syringe with as much dispatch as possible to avoid significant growth during dispensing. The first tube therefore contained $17{\cdot}68/(43{\cdot}6+2{\cdot}5)=0{\cdot}384$ i.u./ml., the second 0·192, etc. When all was prepared the tubes were incubated in a stirred water bath at 30°. At definite time intervals approximating to ½ hr. each set was removed from the water-bath for a few minutes, and pH determinations were carried out on small samples, using a glass electrode. The samples were not returned to the boiling tubes. It was assumed that the small drop in temperature during this short period would not affect different cultures in different ways. In the first experiment six cultures were tested in duplicate, and with the short intervals between the pH measurements this meant that pH determinations were being carried out almost continuously. Partly for this reason the electrode was not washed between samples, but care was taken to proceed from the low pH 'end' of one set to the low pH 'end' of the next set, to work up this set and then to begin the following set at its high pH 'end'. Thus there was at no time a great difference in pH between adjacent samples. Care was taken to standardize the instrument with the buffer at frequent intervals, to renew the potassium chloride junction at still more frequent intervals, and to allow no longer than a moment between samples to diminish the likelihood of fat deposition on the glass electrode. This technique of almost continuous pH measuring seems to have favoured reproducibility in the measurements. After about 5 hr. incubation a marked drop in the pH of the growth control tubes had occurred, and the experiment was concluded.

The second experiment was conducted in a similar manner, except that the experience of the first showed that adequate results could be obtained if the intervals between the measurements were occasionally longer than ½ hr.

RESULTS

These are shown in Figs. 1–12, of which the first six relate to the first experiment. Cultures nos. 607, 924, 799, 495, 804 and 506 were *Str. cremoris*, the rest being *Str. lactis*. Cultures nos. 176, 276, 277 and 278 were capable of producing diacetyl.

Except for the growth controls in Figs. 1–6 each point represents the mean pH of duplicate cultures which showed remarkably close agreement. Out of a total of 361 pairs the differences were less than 0·02 pH, 279 times. Differences of 0·02–0·04 occurred

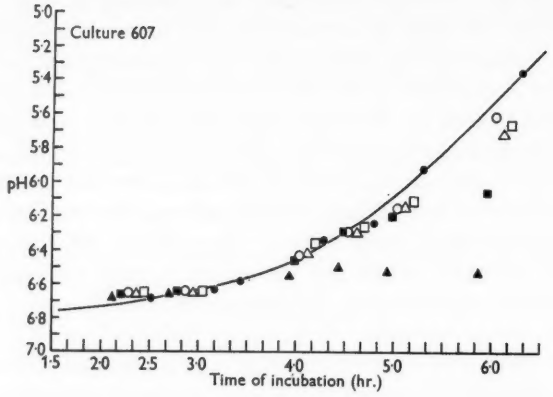

Fig. 1

Figs. 1–6. The rate of acid production in different concentrations of penicillin. Penicillin concentration: ●, zero; ▲, 0·192 i.u./ml.; ■, 0·096 i.u./ml.; ○, 0·048 i.u./ml.; △, 0·024 i.u./ml.; □, 0·012 i.u./ml.

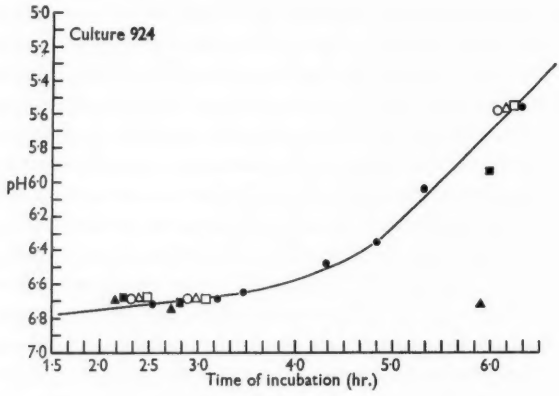

Fig. 2

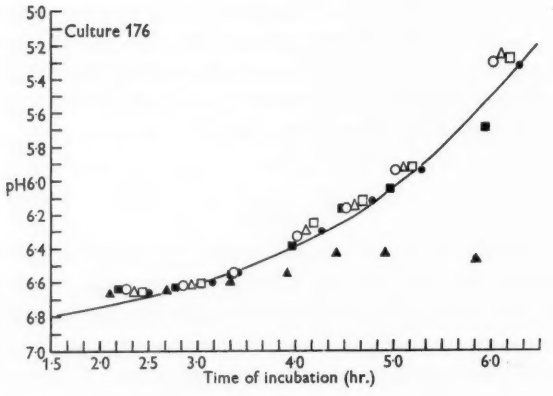

Fig. 3

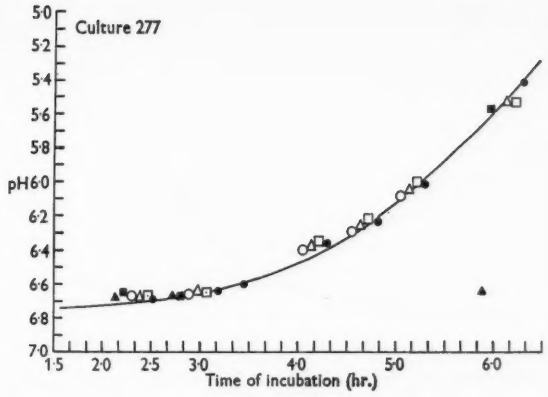

Fig. 4

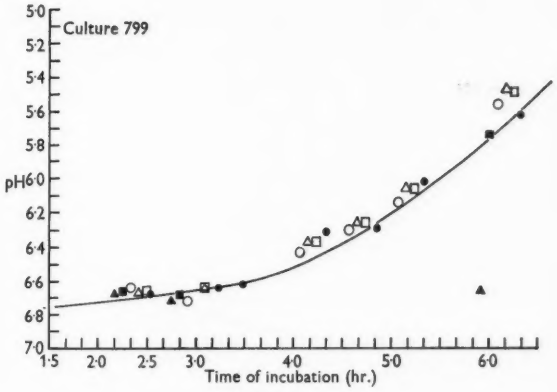

Fig. 5

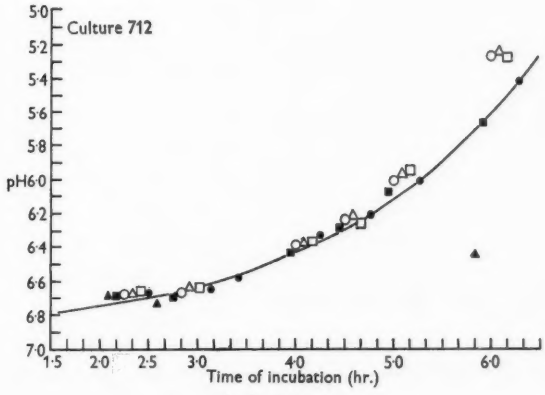

Fig. 6

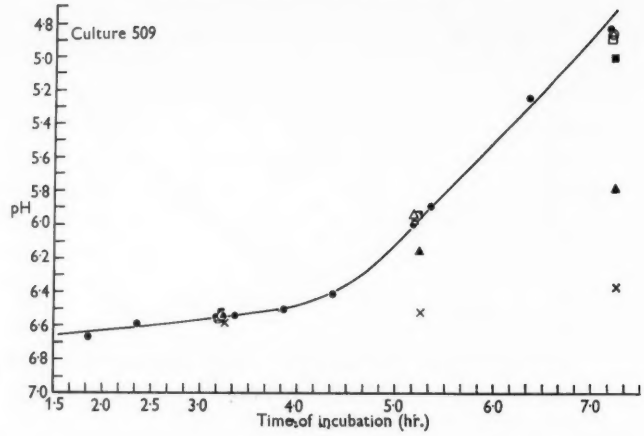

Fig. 7

Figs. 7–12. The rate of acid production in different concentrations of penicillin. Penicillin concentration: ●, zero; ×, 0·384 i.u./ml.; ▲, 0·192 i.u./ml.; ■, 0·096 i.u./ml.; ○, 0·048 i.u./ml.; △, 0·024 i.u./ml.; □, 0·012 i.u./ml.

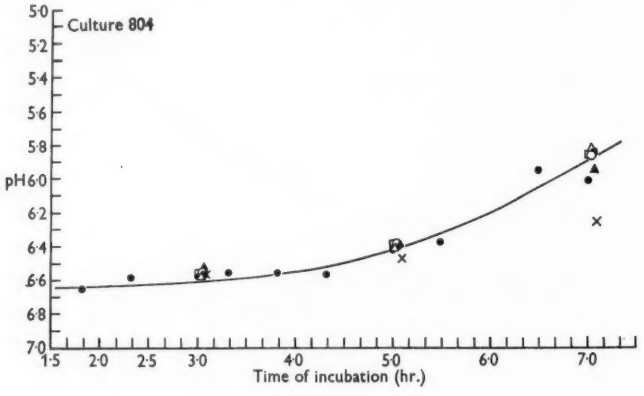

Fig. 8

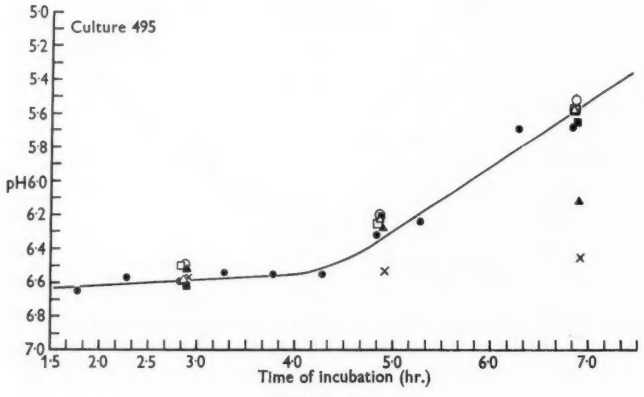

Fig. 9

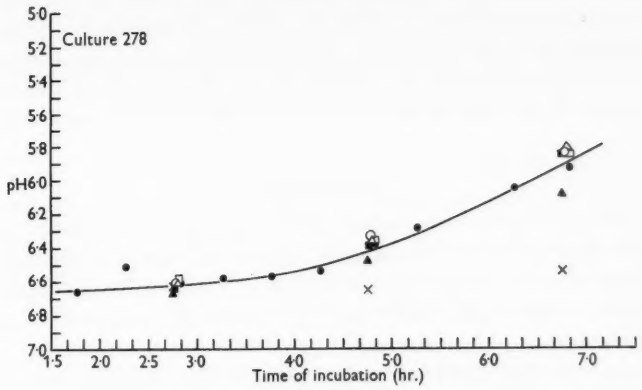

Fig. 10

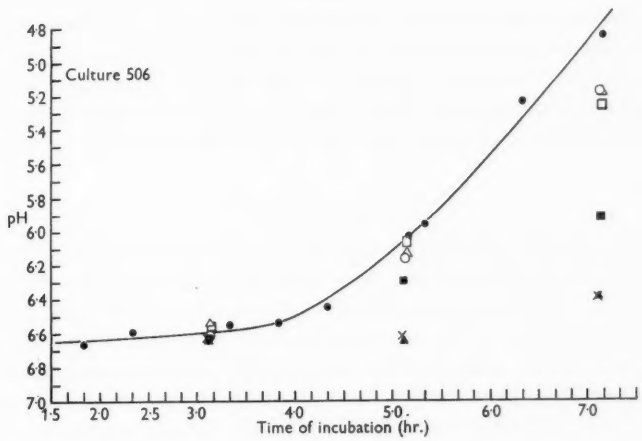

Fig. 11

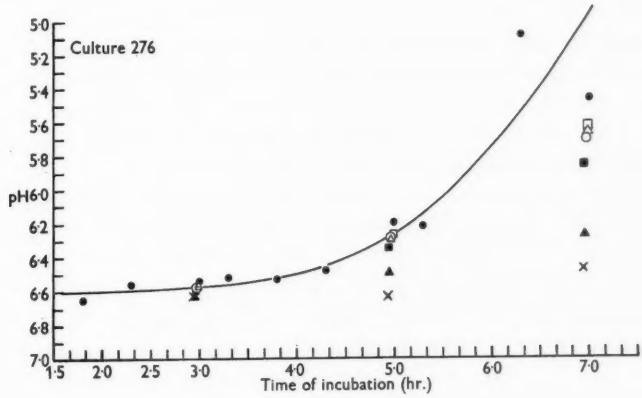

Fig. 12

forty-two times, and differences greater than 0·04, forty times. A difference of 0·04 in pH corresponded to a difference in titratable acidity of about 0·017% lactic acid on a random milk sample at pH values near 5·6 and to 0·006% at pH values near 6·6.

Most of the cultures caused the pH to fall from around 6·7 to about 5 during the course of the experiment. This indicates the production of about 0·54% lactic acid.

DISCUSSION

The different behaviour of different strains of the same species is noteworthy. Thus, for example, while the degree of inhibition of *Str. lactis* 276 follows the penicillin concentration in a regular manner, beginning at the lowest level tested, *Str. lactis* 176 is not inhibited at all until the concentration reaches 0·096 i.u./ml. and even then the effect is only slight.

It will be seen from the figures that three strains of *Str. lactis* and one of *Str. cremoris* are slightly stimulated by low concentrations of penicillin.

From the practical point of view it is necessary to consider the admittedly improbable contingency that low concentrations of penicillin, such as do not affect the starter, will disturb the subsequent ripening of the cheese. In order to test this possibility a series of Cheshire cheese were made in the Institute's Experimental Dairy from milk containing increasing quantities of penicillin. There was no evidence of interference with ripening at concentrations of penicillin which had not diminished starter activity.

CONCLUSION

The amount of penicillin which may be present in milk without interfering with cheese-making depends on the strain of starter in use. Acid production by six of the twelve strains here tested, both *cremoris* and *lactis*, was not delayed until the penicillin concentration exceeded 0·096 i.u./ml. Three strains were slightly inhibited by 0·096 i.u./ml., and the three most sensitive strains, which were slightly inhibited even by 0·012 i.u./ml., suffered a diminution of about 50% in acid production in the presence of 0·096 i.u./ml.

Whitehead (7) and Thomas, Panes & Lewis (8) have recently recorded cheese-making experiments with low concentrations of penicillin. Their results are in general agreement with those reported here.

It is a pleasure to thank Mrs C. Pinnegar for practical assistance and to acknowledge the help of Dr E. I. Garvie in supplying fully active starter cultures.

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637·333·33

644. THE INFLUENCE OF PENICILLIN ON THE MANUFACTURE AND RIPENING OF CHEDDAR CHEESE

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The presence of residual penicillin in milk drawn from cows which have been treated for mastitis has been shown to have an adverse influence when the milk is used for the manufacture of the following types of cheese: Cheddar (1), Cheshire (2), Gruyère (3), Emmenthal (4), Camembert (5, 6), Tilsit (7) and Herrgard (8). It is generally agreed that concentrations of the order of 0·1 unit/ml. have an inhibitory effect on most cheese starters, although different cultures show varying degrees of sensitivity. The nature of the effect on the final mature cheese has not yet been clearly defined. Is the poorer quality sometimes observed due to an interference by the penicillin with some part of the ripening mechanism or is it simply the result of imbalance between moisture and acidity in the cheese brought about by inhibition of starter growth? In 1951–52 a series of experiments were carried out at this Institute in an attempt to throw some light on these questions in the case of Cheddar cheese. This paper gives a brief account of the results.

EXPERIMENTAL METHOD

Cheese was made in two vats, each containing 80 gal. of good quality milk drawn from the same bulk supply and pasteurized at 150° F. (holding period indeterminate but less than 15 sec.). One vat served as a control each day and to the other, immediately before the start of cheese manufacture, was added freshly made aqueous penicillin solution (volume of the order of 50 ml.) calculated to give the required concentration of penicillin in the cheese milk. The penicillin solution was prepared from a weighed quantity of the sodium salt and its concentration was checked by the paper-disk method against *Staphylococcus aureus*.

Throughout the series of experiments the starter used in the cheese vats consisted of equal parts of *Streptococcus cremoris* strains HP and K. Since these starters show a minimum of variation in activity from day to day, provided they do not suffer infection with phage, the control cheese could be made within very narrow time limits and to a predetermined final acidity from day to day. An attempt was made to reach the same acidity in the experimental as in the control curd at two specified stages, viz. (i) at the time that all the whey was drained from the curd, and (ii) at the time of salting the curd. The effect of penicillin could then be expressed as the extra time needed in the experimental vat to reach the normal acidities at 'drying' and 'salting'. In the control vat the normal times were usually about 2 hr. 40 min. from renneting to drying and the same from drying to salting. An 80 lb. cheese was made from the curd in each vat. The cheeses were stored at 55° F. and 82% R.H. for 14 days at which time they were analysed for fat and moisture content, pH values were determined and plugs drawn from them were examined for firmness of body, closeness of texture and normality of flavour. The cheeses were re-examined for general quality after 4 months' storage at 45° F.

During part of the trial a third vat of milk was used to test the effect of additions of penicillinase (penase) as a remedy for the delaying action of penicillin in cheese manufacture. The penase (a trial preparation not normally commercially available) was assayed in milk against known amounts of penicillin with a contact time of 1 hr. at 37° C. The theoretical dose referred to in the experimental results is the amount in any given instance which would destroy a given amount of penicillin completely under these conditions, using the paper-disk assay method with *Staphylococcus aureus*. The freeze-dried penase preparation used was of such strength that an amount of the order of 0·005 g. would inactivate the penicillin present in 80 gal. of milk at a concentration of 0·10 units/ml., under the standard conditions. Since the penase could not readily be used in cheese milk at 37° C. it was to be expected that its efficiency in the cheese vat at approximately 30° C. would be less than the theoretical value would indicate.

RESULTS

Effects of different concentrations of penicillin on the manufacturing process

0·05 *unit/ml.*

On 32 days penicillin was added to the experimental vat of milk at the rate of 0·05 unit/ml. This resulted in an average delay, as compared with the control vat, of 25 min. in reaching a similar acidity in the curd at salting time. The delay on individual days ranged from 5 to 45 min. It is characteristic of penicillin action in the cheese vat that with small amounts present the lag is negligible up to the time of drawing the whey (never more than 5 min.), but that it increases progressively during the cheddaring period. With a presence of 0·05 unit/ml., the total time lag was thus almost entirely during the period 'drying' to 'salting'.

0·10 *unit/ml.*

On 21 days penicillin was added to the experimental vat at the rate of 0·10 unit/ml. This resulted in an average delay of 34 min., with a range from 20 to 55 min. Here again the lag was almost entirely within the 'drying' to 'salting' period.

0·15 *unit/ml.*

On 8 days penicillin was added to the experimental vat at the rate of 0·15 unit/ml. This resulted in a delay of never less than 35 min. On 7 days out of the 8 it proved impossible to reach a normal final acidity in the curd in a reasonable time.

0·2 *unit/ml.*

On 2 days when penicillin was added to cheese milk at the rate of 0·2 unit/ml., the experimental curd developed acid exceedingly slowly and it had to be salted while still 'sweet'. Even in these cases starter activity in the first half of the process was only slightly slower than in the control vat. Soon after the whey was drawn a marked lag set in.

0·5 *unit/ml.*

When, on 1 day, penicillin was added to the milk at the rate of 0·5 unit/ml. the experimental curd was quite 'dead', acid development ceasing soon after the milk was renneted. Even although the curd was held in the vat overnight before salt was added no appreciable development of acid occurred.

CHEESE COMPOSITION AND QUALITY

Since the primary effect of penicillin in cheese milk is to inhibit growth of the starter and hence to reduce rate of acid production, it is to be expected that the moisture content and pH of the final cheese are likely to be abnormally high unless the cheese-maker has applied corrective measures to an adequate degree, e.g. lengthening the time of manufacture to give adequate time for normal acidity to be reached and perhaps increasing the proportion of salt added to the curd. In the experiments described above, the cheese-maker was given freedom to adjust the amount of salt used, if, in his judgement, the control and experimental curds were significantly different in firmness.

0·05 *unit/ml.*

The cheeses made from milk containing 0·05 unit/ml. of penicillin, when analysed 14 days after manufacture, proved to be of normal moisture content and normal pH. Over the whole series the water in the non-fatty substance (W.N.-F.S.) averaged 53·3% for the controls (range 51·6–54·9) and 53·3% for the experimental cheeses (range 51·6–54·5). The pH values of all the cheeses, control and experimental, were within the normal range 4·9–5·0. There was thus no indication that the penicillin, up to this stage in the life of the cheese, had had any effect apart from a slight lengthening of the time of manufacture averaging 25 min. On only one day of this series did the cheese-maker add $\frac{1}{8}$% more salt to the experimental curd. Physical and organoleptic examination of the cheeses did not show any significant differences between the controls and the experimental cheeses, and at a further examination at 4 months all the cheeses were judged to be of first-grade quality. The sole effect of the presence of penicillin in the cheese milk at a level of 0·05 unit/ml. was thus an extension of the time of cheese manufacture of the order of half an hour.

·10 *unit/ml.*

The cheeses made from milk containing 0·10 unit/ml. of penicillin were also not significantly different in moisture content from the controls. The controls averaged 53·6% W.N.-F.S. (range 52·2–55·6) and the experimental cheeses averaged 54·1% W.N.-F.S. (range 52·2–55·7). There were, however, seven instances (out of twenty-one) in which the pH of the experimental cheese had a value over 5·0, and furthermore in this series of experiments the cheese-maker had judged it necessary on nine occasions to add an extra $\frac{1}{8}$% of salt to the experimental curd. The physical and organoleptic examination of the cheeses at 14 days showed that there was a slight tendency to 'pasty' body in some of the experimental cheese, in spite of their normal moisture content. This is difficult to understand. Perhaps the abnormality was due rather to a slightly higher-than-normal pH resulting in a somewhat delayed protein break-down. When the cheeses were examined after 4 months at 45° F., all the controls were judged to be good first-grade cheeses, while the experimental cheeses were in some instances quite normal, and in other instances abnormal in flavour with a 'fermented' or 'yeasty' aroma. In general, therefore, it appeared that a concentration of 0·10 unit/ml. of penicillin in the cheese milk in these experiments was a borderline concentration which even when acidity and moisture seemed to reach normal values led sometimes to production of a somewhat inferior cheese.

0·15 *unit/ml.*

The cheeses made from milk containing 0·15 unit/ml. of penicillin were all definitely abnormal. Even in this series the difference in moisture content between control and experimental cheeses was not significant, perhaps because the longer time occupied in manufacture of the experimental cheeses gave more opportunity for moisture to drain out of the curd even although the moisture-expelling effect of active acid development was absent. All the experimental cheeses had an abnormally high pH, and at both examinations they were 'pasty' in body and 'fermented' or 'yeasty' in flavour. The controls were all good-quality first-grade cheeses. The results indicated that, with the particular starters used, a level of 0·15 unit/ml. of penicillin in cheese milk would always result in the production of low-grade cheese. The few cheeses made from milk containing more than 0·15 unit/ml. of penicillin were all quite abnormal.

EFFECT OF PENICILLINASE (PENASE)

On 20 days during the above-described series of trials a third vat of milk was made into cheese. For this vat addition of the appropriate amount of penicillin solution was followed by an addition of penase solution. After a period, varied in different trials, to allow the penase to act upon and destroy the penicillin, rennet was added to the milk and cheese was made as in the other two vats. The aim in the third vat was to reach the same acidities at 'drying' and 'salting' as in the control vat, the effectiveness of the penase in eliminating the effect of penicillin being judged on a time basis. Throughout these trials attention was concentrated on the question of the commercial practicability of the use of penase. It has been amply demonstrated before that penase will eliminate the effect of penicillin cheese unit given sufficient time of contact at an appropriate temperature *before the addition of starter to the milk*, but under commercial conditions in New Zealand, penase could not conveniently be added to cheese milk before delivery at the factory and any time of contact given after receipt of the milk represents manufacturing time for which an extra labour cost may be incurred. The main question to be settled in the investigation, therefore, was, whether satisfactory penase action in the cheese milk could be achieved in 30 min. or less. The results may be summarized as follows: with a 30 min. contact time, and with concentrations of penicillin ranging from 0·05 to 0·5 unit/ml., amounts of penase equivalent to ten times the theoretical neutralizing amount were necessary to eliminate the effect of the penicillin. With overnight contact at room temperature (impracticable in practice) the theoretical amount was effective in eliminating penicillin action in the cheese milk. Even with ten times the theoretical amount and 30 min. contact, acid production as compared with the control vat was sometimes delayed by 10–30 min., but the delay was not sufficient to cause the cheese to be abnormal in any way.

DISCUSSION

Although the effect of penicillin on different starter cultures varies somewhat, it seems clear from the work of several observers that 0·10 unit/ml. is the critical level in cheese milk beyond which serious effects on final cheese quality may occur. The mechanism involved is apparently an inhibition of acid production, with a resultant abnormally high pH in the cheese and possibly a difficulty in reaching a normal moisture content. In

cheese curd containing penicillin, acid production starts at a normal rate and gradually becomes slower, particularly after the whey has been drawn. The sequence of events is very similar to that which occurs when a trace of bacteriophage active against the starter is present in the cheese curd. In some instances it is impossible to decide whether a slight slowness is due to penicillin or to phage. In the present investigation, there was no evidence of abnormality in the cheese-ripening process which could not be ascribed to abnormalities in pH value. It is possible, however, that the activity of some strains of lactobacilli concerned in the ripening process might be hampered by the presence of penicillin, although Wilkowske & Krienke(9) found that *Lactobacillus casei* was not affected by proportions smaller than 0·3 unit/ml. The main threat of penicillin to cheese quality seems to be that a borderline amount in the cheese milk may not be obvious to the cheese-maker in the absence of a 'control' milk, yet it may result in production of cheese of slightly lower quality than usual.

The use of penase as an antidote to penicillin was not exhaustively investigated. The question of cost could not be considered because the manufacturers of the preparation had not embarked on full scale production and did not propose to do so until the question of usage in commercial practice had been settled. Hence they could not quote a price. Used in a heavy overdose it was evident that penase would neutralize penicillin in a short time in cheese milk, but smaller doses, while effective, would involve a considerable delay owing to the contact time required.

In most dairying countries the whole question of how to deal with antibiotics in cheese milk is still unsettled. Shall the farmer who supplies milk containing penicillin be discouraged from including it in his supply by the application of a heavy monetary penalty? If so, how is the penicillin to be detected? Shall penase be added to all cheese milk to neutralize any penicillin which may be present? How can other antibiotics for which there is no neutralizing enzyme be dealt with? These questions will probably not be settled until much more difficulty in cheese manufacture than has yet occurred lends sufficient urgency to the finding of answers to them.

SUMMARY

The addition of penicillin to cheese milk had the effect of delaying acid production by starter in the cheese curd; any effect on cheese quality could be traced to the delay in acid production and to a high final pH in the cheese. There was no indication of any direct effect of penicillin on the ripening process.

A concentration of 0·10 unit/ml. of penicillin in the cheese milk was a borderline amount with the particular starters which were used. Cheese quality was sometimes adversely affected. A smaller concentration (0·05 unit/ml.) delayed the cheese-making process slightly but had no adverse effect in final cheese quality. Higher concentrations regularly resulted in poor quality cheese.

Penicillinase added to cheese milk neutralized any penicillin present but with a short time of contact of about 30 min., a large excess of penicillinase had to be used.

Our thanks are due to Glaxo Laboratories (N.Z.) Ltd., for kindly supplying the penicillin and penicillinase used in the trials.

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645. LACTOBACILLI ISOLATED FROM THE SURFACE OF NORMAL AND SLIPCOAT STILTON CHEESE

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During an investigation of the surface microflora of Stilton cheese during ripening, Brindley (1) isolated lactobacilli from the surface scrapings of normal and slipcoat cheese. Some of these isolates were sent to the National Institute for Research in Dairying to form part of a collection of lactobacilli which was being built up for classification purposes. During the work on classifying lactobacilli these organisms from Stilton cheese were examined and all but one were serologically grouped and identified by physiological tests. They were also tested against a number of type sera which were available, and it was found that, although isolated from cheese of different origins, most of the strains belonged to the same serological type.

Surface scrapings were taken from Stilton cheese at four different dairies. Three of the samples examined consisted of composite scrapings from twenty to thirty normal cheese; the fourth sample was from an individual slipcoat cheese. At two dairies (V and L) pasteurized milk + starter was used for cheese-making, whereas at the other two (Y and D) raw milk without starter was used.

Thirty-two strains of lactobacilli isolated from the surface of these cheese were examined serologically by precipitin test and grouped according to Sharpe's (2) classification. The strains were also tested by precipitin test against 2 type antisera previously prepared against *Lactobacillus plantarum* 29 (C3) and *L. plantarum* NCTC 6461 (P6), using the methods for making group antisera for lactobacilli (2).

Twenty-six of the strains were serologically grouped as *L. plantarum*, one as *L. casei*, four as *L. brevis*, and one was not identified. Wheater (3), using physiological tests, also identified the same twenty-six strains as *L. plantarum*, and the four strains of *L. brevis* have also since been identified biochemically.

Table 1 shows the serological types of twenty-five of the twenty-six strains of *L. plantarum*. The serological types of the four strains of *L. brevis* were not determined.

The surface lactobacilli isolated from these four cheese consisted mainly of *L. plantarum* and *L. brevis* species. Twenty-four of the strains of *L. plantarum* fell into the same serological type C3, one was type P6, and the other was not identified. Type C3 was thus isolated from cheese made at all four dairies. With the pooled scrapings, taken from twenty to thirty cheese, this serological type must have been present in considerable numbers on the surface of the cheese to have been isolated from all such heterogeneous samples, suggesting that this organism was the predominant lactobacillus on the cheese coats at each dairy. Although only a single slipcoat cheese was examined type C3

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appeared to predominate here also, three out of five of the strains examined belonging to this type.

Examination of further cheese is necessary before it is known whether this particular serological type is common among Stilton cheese. In our experience, however, serological types of lactobacilli encountered in cheese, even from the same dairy, are likely to be numerous and varied. The finding of the same organism on cheese from four different dairies in the same region strongly suggests a common source.

Table 1. *Serological types of* Lactobacillus plantarum *isolated from surface scrapings of Stilton cheese*

Dairy	Method of cheese-making	Type of cheese	No. of strains of lactobacilli examined	Serological types of *L. plantarum*		Identity of other strains
				C3	P6	
V	Past. milk + starter	Normal	20	18	1	1 *L. brevis*
L	Past. milk + starter	Normal	4	1	0	1 *L. casei*, 1 unknown, 1 *L. plantarum* of unknown type
Y	Raw milk	Normal	3	2	0	1 *L. brevis*
D	Raw milk	Slipcoat	5	3	0	2 *L. brevis*
Total numbers of strains			32	24	1	7 —

Such a source, however, was not traced to the milk. There was a common supply of milk for dairies V and Y, the milk being collected from the same district, but the other two dairies L and D obtained their milk from quite different areas. The dairies themselves were some distance apart, L being 55 miles, D 18 miles and Y 7 miles respectively from V. There are, however, many other ways in which such an organism could be distributed: dairy L supplied mature cheese to dairies V and D, and dairy Y occasionally supplied cheese to D, so that there was some communication between them. No lactobacilli were isolated from eight mature cheese coats examined, so that it is more likely that the organisms would be transferred on adventitious material rather than on the coats of ripe cheese. The fact that a particular lactobacillus was common to several dairies indicates the possibility of defect-causing or pathogenic bacteria also being similarly disseminated.

It is of interest that whatever the conditions causing slipcoat, these appear to have no effect on the species and types of lactobacilli present on the cheese coats; *L. plantarum*, including type C3, and *L. brevis* were found on both normal and slipcoat cheese. The pH's of the cheese coats also had little effect on the species and types present (pH's of scrapings being V = pH 5·5, L = pH 6·7, Y = pH 6·2, D = pH 5·8), although the lactobacillus flora appeared to be slightly more varied with L which had a higher pH than the others.

The serological type C3 has previously been isolated twice by us, once from an oral source and once from Cheddar cheese.

We wish to thank Dr A. T. R. Mattick for his interest in this work.

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646. THE STEAM STRIPPING OF TAINTS FROM LIQUIDS

V. THE BASIC DESIGN OF CREAM DEODORIZING EQUIPMENT

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(With 11 Figures)

INTRODUCTION

Previous papers in this series[2,3,4,5] have shown the fundamental characteristics of a number of types of deodorizing plant in which steam is used to carry away portions of the volatile tainting substances found in cream. Particular studies have been made of units in which cream and steam mix in co-current flow, flash steam units (or units in which steam is boiled from the cream), multiple-stage mixing units, mixing units with counterflow connexions, batch systems, and continuous counterflow columns.

This present paper shows how the basic data already presented can be used for the design of deodorizing equipment. Alternatively, the effect on volatile flavour components can be indicated for proposed or existing plant, although the greatest value of the basic data cannot be realized until more is known about the amounts and volatilities of the desirable and undesirable flavouring substances in cream used for butter-making.

DEODORIZING PRINCIPLES

The stripping factor

The performance of equipment for deodorizing cream by steam stripping of the taints is best studied by means of the stripping factor which in this series has been given the symbol F and is the value of the dimensionless group mV/L. The effect of this group on plant performance has been studied in previous parts of this series and it is therefore advisable to have a clear physical meaning of the above dimensionless number.

Fig. 1*a* shows in block form a simple separation process in which 100 lb. of water containing 10 p.p.m. of some solute is mixed with 100 lb. of solute-free water to give 200 lb. of water containing 5 p.p.m. of solute from which a 100 lb. product could be taken. This added solvent has the same affinity for the solute as the original water. The ratio of this added water (of the same affinity) to the original water provides the stripping factor, F, which is unity in this case.

If an immiscible added solvent such as kerosene were used (which we can assume has a threefold greater affinity for the solute than has water) then 100 lb. of water mixed with 100 lb. of kerosene would cause a redistribution of the solute such that 2·5 p.p.m. remained in the water and three times this amount (7·5 p.p.m.) passed into the kerosene. The partition coefficient of 3 allows 1 lb. of kerosene to retain at equilibrium as much solute as 3 lb. of water, and the system would therefore act as shown in Fig. 1*b*. Since 1 lb. of kerosene has the equivalent solute holding capacity of 3 lb. of water, the stripping factor is $3 \times 1/1 = 3$ and the reduction in solute concentration is $1/(1+3) = 1/4$ of original

value. Similarly, V lb. of steam (the solute in the steam having an equilibrium coefficient of m) will function as mV lb. of liquid and when applied to L lb. of liquid the stripping factor for a mixing type system must be $F=mV/L$ and the ratio of final and initial concentrations is $1/(1+F)$ as derived in Part I.

The stripping factor acts therefore as a dilution factor, the appropriate reasoning for continuous countercurrent flow having been presented in Part IV.

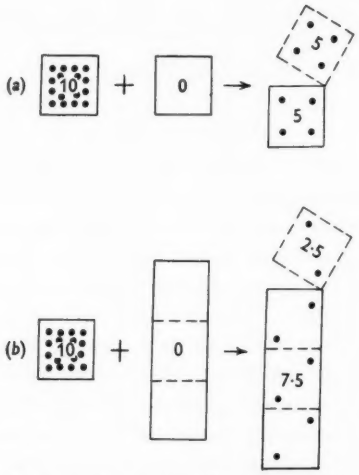

Fig. 1. A simple representation of a separation process. Separation is shown to be an example of dilution; thus (*a*) shows the effect of adding an equal weight of water to an aqueous solution, and (*b*) shows the effect of adding a fluid having three times the affinity for the solute than has water.

Comparison of operating curves

Fig. 2 of Part II (3) shows the percentage taint remaining as a function of the stripping factor for a number of mixing type systems. The form of the curves suggests that taint removal is slow under conditions of a high value of stripping factor, but it should be realized that the reduction in taint concentration is here being compared with the initial concentration. The butter-maker is interested more in the taint concentration remaining in the treated cream and the usefulness of the operating curves would be increased by a graphical plot which suppressed the scale for taint concentrations near 100% but expanded the scale to show operating conditions for high values of the stripping factor. Graph paper scaled logarithmically is very suitable for this purpose, and Fig. 2 shows curves from fig. 2 of Part II replotted on logarithmic paper.

A 10 to 1 reduction from 100 to 10 or from 10 to 1 or from 1 to 0·1 measures the same distance in inches; this property being general: a vertical or horizontal measurement in inches represents the same percentage change in values on any part of the graph. The wide spread among the curves at the bottom right-hand corner of the graph shows, in particular, that multiple-contact and multiple-contact-counterflow apparatus give a large steam saving compared with single-stage operation when the value of the stripping factor is high. The percentage difference in steam consumption between any two systems can be obtained directly by measurement of the horizontal distance between two curves

and comparing this measurement with a portion of the logarithmic scale. A similar procedure can be used on the vertical scale for comparing relative taint concentrations for equal values of steam flow.

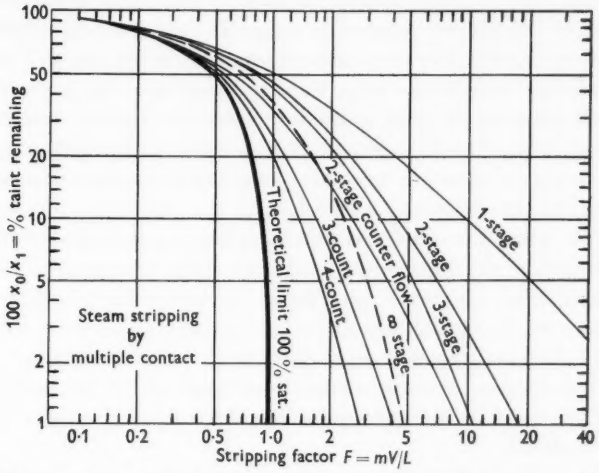

Fig. 2. The ratios of final to initial concentrations of taint for multiple-contact systems plotted (as percentage values) against values of the stripping factor. The limiting curve for multiple-contact systems is shown dotted and characterizes an infinite number of stages. The limiting curve for any form of counterflow is the 100% saturation curve.

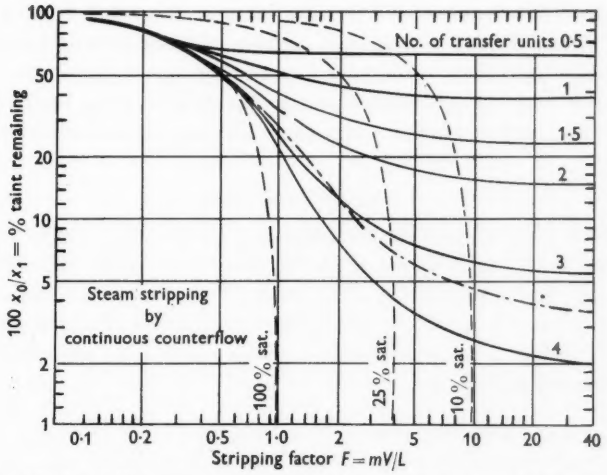

Fig. 3. The ratios of final to initial concentrations of taint for continuous counterflow systems are plotted (as percentage values) against values of the stripping factor. Full lines are for rate calculations based on the transfer concept. Dotted lines show the saturation of the outgoing steam as a percentage of maximum possible in a simple system. The chain line shows values where $(HTU)_L = 4{\cdot}7 + 1{\cdot}3(1/F)$.

The full lines of Fig. 3 show a plot (on a similar logarithmic scale) of the continuous counterflow results replotted from fig. 3 of Part IV (5). A comparison shows distinctly the difference in operating characteristics between mixing type plant as represented by Fig. 2,

and continuous counterflow plant as represented by Fig. 3. It is worth reiterating here that both of these figures are based on equations of a general nature derived from fundamental considerations. In Fig. 2 the value of m is the ratio between the concentration of taint in the steam and the concentration of taint in the liquid at the point where the steam-liquid mixture begins the separation stage. If the vapour-liquid taint concentrations have attained equilibrium or near equilibrium then the value of m to use is the equilibrium coefficient for the particular taint in the system. If, however, equilibrium has not been attained (by insufficient time of contact or lack of intimacy of contact) then the value of m to use will be some fractional value of the equilibrium coefficient.

Increasing amounts of steam or increasing values of the equilibrium coefficient cause increasing values of the stripping factor which in the case of mixing-type deodorizing plant continues to cause the solute concentration level to diminish, but for the case of continuous counterflow plant the solute level is normally asymptotic to some level. All volatile flavouring solutes in cream must follow the characteristics shown in Figures 2 and 3 and since some flavouring substances are desirable and some are undesirable the flavour components remaining in a deodorized cream must be different from plants of the mixing type and the counterflow column type. Presumably the amount of each type will depend on the number, kind and volatility of the flavouring substances as well as our taste classifications.

The chain line in Fig. 3 is for a particular column dealt with in Part IV in which the Height of a Transfer Unit in feet is equal to $4{\cdot}7+1{\cdot}3(1/F)$, where 4·7 is the HTU for the liquid side and 1·3 is the *HTU* for the steam side. This column will have but one or two transfer units at low values of the stripping factor, is equal to 3 transfer units when $F=2$, and gradually increases to 3·5 transfer units at $F=40$. A column with a lesser liquid side *HTU* would result in a greater number of transfer units where F was large and the tail of the dotted line would have a more pronounced downward trend. For all practical purposes it would seem then that the column with a low liquid side resistance would give flavour components not unlike those for multiple-contact counterflow plant.

Degree of saturation

In Fig. 4, let the block diagram represent any deodorizing system (having neither condensation nor flash steam) with a single supply of fresh steam. The concentration of taint in the steam cannot exceed the equilibrium value equal to mx_1, hence the fraction of saturation is given by y_0/mx_1, where y_0 is the actual concentration of taint in the outgoing steam. From a material balance $y_0=(x_1-x_0)/(V/L)$ and therefore the percentage of saturation is given by

$$100\frac{y_0}{mx_1}=100\frac{x_1-x_0}{x_1}\frac{1}{mV/L}=100\frac{1-(x_0/x_1)}{F}.$$

For any assumed values of the degree of saturation of the taint in the outgoing steam as parameter, values of x_0/x_1 can be calculated for values of F, the dotted curves in Fig. 3 being for cases of 100, 25 and 10% of saturation. The intersection of a working point with a curve of this family will show directly the degree of saturation obtaining. The line for 100% saturation can be obtained theoretically in a continuous counterflow column of great height or one having low resistances to transfer of taint, and it is of particular interest as it represents the theoretical maximum efficiency which could be

obtained from any simple system disregarding heat recovery or other added equipment. The 100% line is shown also in Fig. 2.

For short height counterflow columns of either the wetted wall or spray type the quantity of taint transferred from the liquid across the resistances in a short time-interval is relatively small. As the rate of steam flow increases, a decreased degree of saturation does not necessarily take place, because the combined effects of a decreased steam side resistance due to the greater steam flow, and a decreased liquid side resistance due to mechanical effects of the steam velocity in the liquid, can increase the rate of taint transfer and a nearly constant degree of saturation could result particularly in the range of x_0/x_1, between 40 and 100%.

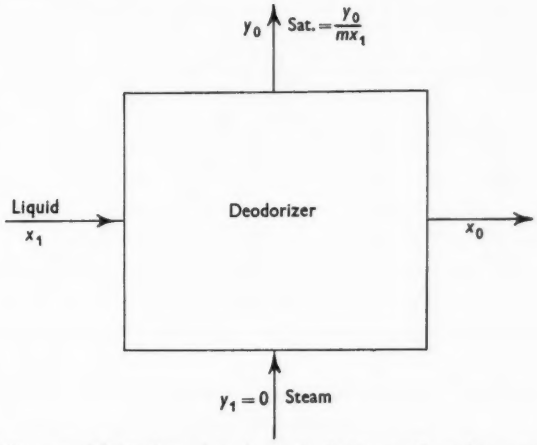

Fig. 4. Block diagram of deodorizer. Any simple deodorizing unit can be represented by the block diagram as illustrated.

Multi-component stripping

The extent that a solute is removed from a liquid mixture undergoing a steam stripping process depends on the type of plant, the amount of steam used, and the volatility of the solute. The amounts of flavouring substances present in a treated cream will, in addition, depend on the original concentrations of the flavouring substances. The final array of desirable and undesirable flavouring components in a treated cream will thus depend on processing conditions as well as the physical properties of the substance in cream.

In the design and operation of deodorizing equipment, consideration must be given to the effect on flavour components by a change in conditions. A decrease in deodorization can be accomplished either by reducing the number of vessels in a multi-vessel plant or by reducing the rate of steam flow. The effect of these changes on solutes of different volatilities can be calculated by means of Figs. 2 and 3. Fig. 5 extends the range of values given in Fig. 2 so that solute concentrations can be determined for high values of the stripping factor.

Tables 1–3 have been calculated, based on a constant percentage removal of a medium volatile substance of $m=10$. The residual concentrations (as percentage of initial concentrations) of a substance having one-fifth this volatility (i.e. $m=2$) and a substance having five times this volatility (i.e. $m=50$) have been calculated.

In Table 1 if we take, by way of example, the medium volatile to be a taint and the high volatile a desirable flavouring substance, an increase in final taint-level from 3·9 to 10 will increase the flavouring substance about 12-fold if two stages are used instead of three. A similar increase in final taint level by reducing the steam raises the flavouring substance level about threefold only. Similarly, in Table 2 for continuous counterflow

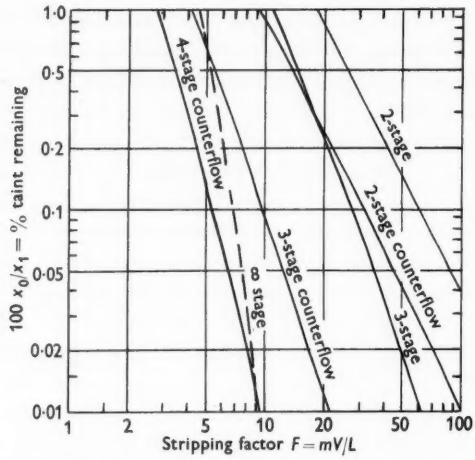

Fig. 5. Extension of characteristics shown in Fig. 2. Low values of residual taint levels are shown.

Table 1. *Residual concentration ratios for countercurrent mixing type plant*

Number of vessels	Steam used (lb./lb. liquid)	Residual concentration as percentage of initial value: Volatility of solute Low $m=2$ (%)	Medium $m=10$ (%)	High $m=50$ (%)
3	0·25	53	3·9	0·046
2	0·25	57	10·0	0·57
3	0·17	68	10·0	0·14

Table 2. *Residual concentration ratios for continuous counterflow current*

Number of transfer units	Steam used (lb./lb. liquid)	Residual concentration as percentage of initial value: Low $m=2$ (%)	Medium $m=10$ (%)	High $m=50$ (%)
4	0·27	48	5·3	2·4
3	0·27	49	10·0	5·8
4	0·16	67	10·0	2·7

plant a rise in final taint level from 5·3 to 10 by a decrease in transfer units (e.g. a decrease in the number of vessels could achieve this) the flavouring substance level would increase twofold, but a similar adjustment in taint level by a reduction in steam would cause little change in flavouring substance concentration. The economics of a saving in steam must be balanced against the change in flavour components as judged by taste. It is important to note that mixing type units can reduce a high volatile component to a very low concentration, a characteristic which may be undesirable if the high volatile solute improves

the taste or aroma of a resulting butter, but desirable if some objectionable taint is highly volatile.

The effect of the design of deodorizing equipment on flavour components can be illustrated in Table 3, which is based upon the reduction of a solute of $m = 10$ to 10% of its initial value in all cases. The amounts of steam necessary to achieve this result can be compared and indicate the relative efficiencies of the plants listed. A co-existing solute

Table 3. *Stripping efficiency as affected by type of plant and solute*

Type of plant	Steam used for deodorizing (lb./lb. liquid)	Residual concentration as percentage of initial value. Volatility of solute: Low $m=2$ (%)	Medium $m=10$ (%)	High $m=50$ (%)
Multiple contact				
Single contact	0·90	36	10	2·17
Double contact	0·43	49	10	0·72
Triple contact	0·35	53	10	0·30
Infinite contact	0·23	63	10	0·001
Multiple-contact countercurrent				
Double contact	0·25	57	10	0·59
Triple contact	0·17	68	10	0·21
Quadruple contact	0·14	72	10	0·035
Continuous countercurrent ($HTU)_l = 4·7$, ($HTU)_g = 1·3$ (chain line in Fig. 3)	0·25	52	10	4·30
100% saturation of low volatile	0·09	51	10	—

of $m = 50$ will be reduced to proportions of the initial 100% value as shown in the final column. The range of values shows that the various types of plant will produce differences in flavour components whenever there are differences in volatilities of the component solutes. In general for mixing type plant, the more efficient the plant the lower the final concentration of high volatile solute; especially this is so for an infinite contact plant which in practice can be obtained by a batch process (see Part III). For the same degree of removal of a low volatile solute, continuous countercurrent plant will often give a cream having flavour components with a greater proportion of high volatile solutes than the components from cream treated in a multiple-contact plant. In practice, differences in vacua between vessels cause condensation or flashing of steam which modifies the figures given in Table 3, although the general conclusions are not usually altered. Condensation in the vessels of some plant can increase residual values, particularly of high volatile solutes.

In general, any plant which requires a comparatively large steam-flow will reduce a low volatile substance by a greater amount than a plant designed to use a small amount of steam. Continuous counterflow stripping tends to greater effectiveness in the removal of low volatile substances. It should be noted here that the flavour components in butter will depend not only on the flavour components in the cream, but also on their distribution between the fat phase and the serum phase.

Several other methods of plotting are useful in the study of steam-stripping plant. A plot of the concentration ratio x_0/x_1 on a logarithmic scale against a natural scale for the stripping factor is of special value when analysing the results of batch processes or

comparing the limits of multiple contact plant. On this type of plot the function $x_0/x_1 = e^{-F}$ gives a straight line which can be used to find values of m in experimental work. Another useful method is to plot the value of x_1/x_0 (i.e. the reciprocal of x_0/x_1) against F or $1+F$, this method being particularly valuable for plotting results of simple single contact operations where a straight line plot results: in experimental work a plot of x_1/x_0 against V/L will give a straight line in which the slope indicates the value of m since $F = mV/L$.

PLANT ANALYSIS

Hypothetical design 'Taint'

As for other chemical engineering plant, a design for separation of one component from a liquid mixture requires some knowledge of the distribution constants for the component in the particular system, and some knowledge is also required of the initial amount present and the amount to remain in the stripped liquid. With cream this poses a difficult problem because in general none of the above requirements are known. For design purposes the present author uses a hypothetical taint having an equilibrium coefficient of 10 in cream of 40% fat content. The value of 10 represents a volatility in steam which is somewhat greater than that for acetoin but less than that for diacetyl, experience in cream treatment plant indicating that prevailing taints are probably in this category. Further, the hypothesis is made that in areas where taints are known to be severe, the final concentration of this hypothetical taint remaining in the cream is to be 10% of the initial concentration. For areas where taint conditions are known to be less severe the final taint concentration is taken as 25% of the initial value. No doubt other combinations of assumed volatilities and degrees of separation would be useful, and it may well be that in countries other than New Zealand a different set of design conditions are more appropriate.

Use of basic equations for multiple-contact design

The basic equations developed in Part I (2) for application to mixing or concurrent type deodorizing plant can be used with the hypothetical taint for the design of plant or to compare the performances of plant. The following example illustrates the method applied to a problem in which the steam consumption of a particular deodorizing equipment is required.

A Triple Vacreator without a countercurrent connexion (see fig. 3, Part II) is required to deodorize cream at a rate of 12,500 lb./hr. and 90% of the taint (having an equilibrium coefficient $m = 10$) is to be removed.

Temperature of cream entering no. 1 vessel	145° F.
Vacuum in separating vessels nos. 1 and 2	13 in.
Corresponding saturation temperature	185° F.
Vacuum in flash cooler	26 in.
Corresponding saturation temperature	125° F.

The 12,500 lb./hr. of cream is heated by direct steam contact in the first vessel from 145 to 185° F. so that the cream flow leaving vessels 1 or 2 is 13,000 lb./hr.

In the flash cooler this flow of cream is cooled from 185 to 125° F. with the formation

and rejection of 650 lb./hr. of flash steam. The general equation for the fractional change in taint concentration per stage (equation A 2·2, Part I) is

$$\frac{x_0}{x_1}=\frac{1+Gy_1/Lx_1}{Q+F},$$

and if $y_1=0$, becomes $1/(Q+F)$ or $1/(1+F)$ for no condensation or flash steam

$$\frac{x_c}{x_b}=\frac{1}{Q+F}=\frac{1}{\left(1-\frac{650}{13{,}000}\right)+\frac{10\times 650}{13{,}000}}=\frac{1}{0{\cdot}95+0{\cdot}5}=\frac{1}{1{\cdot}45},$$

thus x_b, the taint concentration in the cream leaving the second vessel, must be 14·5% since x_c the final taint level is to be 10%.

Assume 2000 lb./hr. of steam is rejected from each of the first two vessels. Then from the equation,

$$\frac{x_b}{x_a}=\frac{1}{1+F}=\frac{1}{1+\frac{10\times 2000}{13{,}000}}=\frac{1}{1{\cdot}0+1{\cdot}54}=\frac{1}{2{\cdot}54}.$$

Thus x_a, the taint level in the cream leaving the first unit, is $14{\cdot}5\%\times 2{\cdot}54=36{\cdot}8\%$. Similarly,

$$\frac{x_a}{x_1}=\frac{1}{Q+F}+\frac{1}{\left(1+\frac{500}{12{,}500}\right)+\frac{10\times 2000}{12{,}500}}=\frac{1}{1{\cdot}04+1{\cdot}6}=\frac{1}{2{\cdot}64}.$$

Thus x_1 is $36{\cdot}8\%\times 2{\cdot}64=97{\cdot}4\%$.

Since the initial taint level is taken as 100%, the assumed steam of 2000 lb./hr. per vessel is slightly low and a new trial of, say, 2050 lb./hr. could be made.

$$\text{Deodorizing steam } \frac{4000}{1250}=3{\cdot}2 \text{ lb./gal. cream.}$$

$$\text{Total steam } \frac{4500}{1250}=3{\cdot}6 \text{ lb./gal. cream.}$$

The average in practice for the assumed conditions is about 3·8 lb./gal. Table 4 shows the results of the method applied to other Vacreator systems.

Table 4. *Steam demands for conventional Vacreator systems*

(Based on cream preheated to 145° F. by waste heat.)

Type of plant	Final taint level (as % of original)	Cream flow (G.P.H.)	Calculated steam per gallon cream (lb./gal.)	Typical steam per gallon in practice (lb./gal.)
Three-leg Vacreator	10	1250	3·60	3·8
Five-leg Vacreator	10	1700	3·08	3·1
Split-flow Vacreator	10	1500	3·75	3·8
Tandem Vacreator	12·5	600	4·90	5·0
Tandem Vacreator	25	800	2·15	2·3

Tandem Vacreator: consists of a steam section plus a cooling section. Often used where taint levels in cream are not high and cream flows not large. Three-leg Vacreator: consists of two steam sections followed by one cooling section. Five-leg Vacreator: consists of three steam sections followed by two cooling sections. Split-flow Vacreator: consists of one steam section from which the cream flow divides into two Tandem units in parallel.

The agreement between the calculated values and the typical practical values gives confidence in the method. The Tandem Vacreator cannot reduce the hypothetical taint to a 10% level unless the cream flow is reduced below 600 gal./hr.

Use of cooling section

The use of the vacuum-cooling vessel has been criticized from time to time as being unnecessary. However, its continued use is warranted because it performs the following desirable functions:

(*a*) Gives rapid cooling of the cream.
(*b*) Removes water condensed into the cream.
(*c*) Reduces the percentage of small-size globules or fat clumps.
(*d*) Removes a portion of the taint in the cream.

The last function, that of a deodorizer (it is obviously a case of equilibrium distillation) warrants further study. From the worked example it can be seen that a steam section need reduce the taint level to 14·5% only instead of 10% when a flash-cooling vessel is used. Owing to the nature of the curves this reflects in a considerably reduced value for the stripping factor and the required steam. Table 5 shows that a Tandem Vacreator can reduce the hypothetical taint level to 25% with 2·15 lb. of steam per gallon. Without a flash-cooling vessel this figure would be 3·35, and therefore a saving of 1·2 lb./gal. would be made, whereas with cream cooled over the same range using regenerative cooling by incoming cream, the steam saving would be only 0·54 lb./gal. Regenerative cooling down to 75° would, however, recover 1·0 lb. steam per gallon of cream.

The improved efficiency given to a deodorizing plant by final vacuum cooling can be further increased by the use of a greater degree of vacuum. Thus if it were possible to maintain a vacuum of 29·25 in. the cream would be cooled to 70° F. and the necessary steam would, in the above example, be reduced to 1·46 lb./gal. Thus flash-cooling results in greater steam economy than direct heat recovery by regeneration.

Table 5. *Heat economy obtained by use of vacuum flash cooling*

(Cooling ranges 185 to 125° F. and 185 to 70.)

Type of plant	Residual taint as percentage of original	Steam consumption in lb./gal. of cream: No flash cooling, temp. 185° F.	Present flash cooling, temp. 125° F.	Flash cooling at high vacuum, temp. 70° F.
Tandem Vacreator	25	3·35	2·15	1·46
Tandem Vacreator	20	4·36	2·86	2·00
Tandem Vacreator	15	6·02	4·01	2·86
Three-leg Vacreator	10	4·78	3·60	3·00
Steam saving by regenerative cooling to same temperatures	—	—	0·54	1·00

The economy obtained by flash cooling depends on the type of Vacreator plant and the final taint level desired. These points are illustrated in Table 5.

Use of basic equations for multiple contact countercurrect design. (See Fig. 3, Part II)

The example already given for multiple contact design can be reworked but operating vacua are slightly different in this case. Thus:

Cream flow	12,500 lb./hr.
Preheat temperature	145° F.
Vacuum in no. 1 vessel	15·5 in.
Corresponding temperature	178° F.
Vacuum in no. 2 vessel	9·5 in.
Corresponding temperature	194° F.
Vacuum in flash cooler	26·0 in.

Due to condensation, the cream flow leaving the first unit is 12,950 lb./hr., and leaving the second unit is 13,400 lb./hr. If we assume a steam flow into the second unit of 2850 lb./hr. then the flow to number one will be 2650 lb./hr. and the steam flow leaving the first unit will be 2250 lb./hr.

Due to the higher operating temperature in vessel 2, the cream enters the cooling vessel at 194° F. forming 800 lb./hr. of steam.

For the cooling vessel,

$$\frac{x_0}{x_b}=\frac{1}{Q+F}=\frac{1}{\left(1-\frac{800}{13{,}400}\right)+\frac{10\times 800}{13{,}400}}=\frac{1}{0{\cdot}94+0{\cdot}596}=\frac{1}{1{\cdot}536},$$

and the steam sections need therefore reduce the taint level to 15·4 %.

For vessel 2,

$$\frac{x_b}{x_a}=\frac{1}{Q+F}=\frac{1}{\left(1+\frac{200}{12{,}9650}\right)+\frac{10\times 2650}{12{,}950}}=\frac{1}{1{\cdot}015+2{\cdot}05}=\frac{1}{3{\cdot}065},$$

and $x_a=3{\cdot}065\times 15{\cdot}4=47{\cdot}3\,\%$.

For vessel 1,

$$\frac{x_a}{x_1}=\frac{1+\frac{Gy_1}{Lx_1}}{Q+F}=\frac{1+\frac{2650\times 10\times 15{\cdot}4}{12{,}500\times 100}}{1{\cdot}032+\frac{10\times 2250}{12{,}500}}\quad(\text{since } y_1=mx_b)$$

$$=\frac{1{\cdot}326}{2{\cdot}832}=\frac{1}{2{\cdot}14}.$$

Thus $x_1=2{\cdot}14\times 47{\cdot}3\,\%=102\,\%$, which is close enough to the assumed 100 %. Thus the assumed figure of 2850 lb./hr. of steam entering the system is correct.

$$\text{Deodorizing steam}=\frac{2250}{1250}=1{\cdot}80 \text{ lb./gal. cream.}$$

$$\text{Total steam}=\frac{2850}{1250}=2{\cdot}28 \text{ lb./gal. cream.}$$

Similar calculations have been made for the other applications of multiple contact counterflow Vacreator systems, the results being set out in Table 6 with a column to show the percentage saving over the unchanged Vacreator based on the figures given in Table 4.

Table 6. *Steam demand for Vacreators changed to multiple-contact counterflow operation*

(Based on cream heated to 145° F. by waste heat.)

Type of plant	Final taint level (%)	Cream flow (G.P.H.)	Calculated steam (lb./gal.)	Typical steam in practice (lb./gal.)	Calculated steam reduction* (%)
Three-leg Vacreator	10	1250	2·28	2·40	36·7
Five-leg Vacreator	10	1700	1·72	1·75	44·1
Tandem Vacreator	25	800	1·25	Insufficient data	58·1

* Comparison is made here with similar plant as normally operated without the counterflow connexion.

Reference substances and plant comparisons

The direct comparison of the efficiencies of two types of deodorizing plant is best done in the field using portions of the same cream, but as this is not always possible, recourse has to be made to comparisons by use of reference substances. Most comparative work of this nature has been done with diacetyl which has an equilibrium coefficient in water of about 39 at low concentrations. As a natural constituent of cream and butter diacetyl is very suitable for use as a reference substance and has value for investigations of the general performance of any one particular deodorizing plant, but for plant comparisons a highly volatile reference substance does not seem to be desirable as shown by the figures in Table 3, where a comparison based on equal removal of a substance of $m=50$ would give different steam ratios to those listed which are based on a substance where $m=10$ for New Zealand conditions. Use has been made of acetoin as a reference substance also, but it suffers in this respect by being of low volatility.

Tables 4 and 6 show that there is agreement between calculated figures based on $m=10$ and practical figures for steam usage. Calculations given in Part IV for continuous counterflow equipment and based on a value of $m=10$ also give figures which appear to be in accord with practice, but it should be noted again here that the design of a continuous counterflow deodorizer based solely on a solute of $m=10$ may not give satisfactory deodorization when a solute of high volatility occurs in practice. Either a hypothetical reference substance having an equilibrium coefficient considerably greater than 10 should be employed or, better, a minimum number of liquid side transfer units should be specified.

A deodorizing plant which has proven satisfactory in one country may not be satisfactory in another, due to different taints and taint intensities. The ability to remove taint and the efficiency of the plant can be assessed by the methods set out in this paper, a task which is especially easy if the design of the deodorizer is based on co-current mixing, flash-steam production or boiling of the cream under vacuum.

Continuous counterflow plant

The effects of liquid and vapour side resistances on overall column performance have been discussed in Part IV (5), but more specific cases are illustrated in Fig. 6 for an overall height of a transfer unit of $(HTU)_L=4+4/F$, and for column heights of 2, 8 and 16 ft. For purposes of comparison the curve of $n=4$ is shown as it represents a case where there is no resistance to transfer of taint on the steam side for a column 16 ft. high. Similarly, if there were no liquid side resistance, $(HTU)_L$ would equal $4/F$, and for a column 16 ft. high n would be $4F$, the appropriate curve having been calculated and plotted.

The points on curve H of Fig. 6 were obtained experimentally from a counterflow section 36 in. high and 5 in. diameter, using a 10,000 lb./hr. spray of water ($\frac{5}{64}$ in. holes) containing diacetyl. Similarly, curve I shows points from a counterflow column 36 in. high and 13 in. diameter using a 1000 lb./hr. spray of water ($\frac{1}{8}$in. holes) containing diacetyl. In both cases the tendency is to follow more the characteristic of a constant degree of saturation rather than the characteristic due to constant heights of transfer units, $(HTU)_l$ and $(HTU)_g$. The positions of the curves are such that the steam-side resistance can have only a small effect and the rapid reduction of diacetyl concentration with increasing

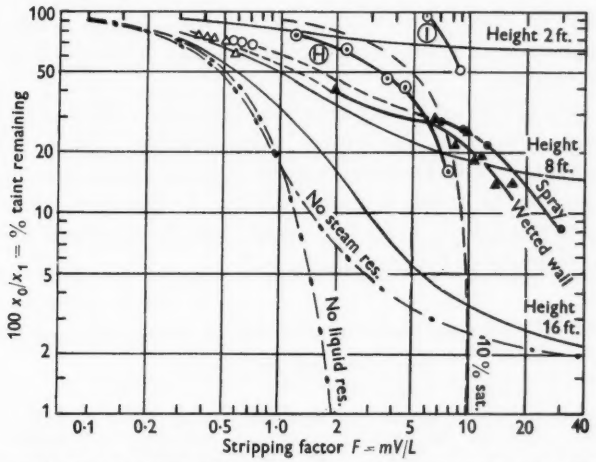

Fig. 6. The fundamental effect of column height. Illustrated by the curves for 2, 8 and 16 ft. for a case where $(HTU)_l = 4$ ft. $= (HTU)_g$. The characteristics are also shown for cases when either the liquid side resistance or the steam side resistance are zero. Curves H and I show that mechanical disruption of the liquid is an important effect especially for short columns. Values of $m = 37{\cdot}5$; liquid rate 10,000 lb./hr. for H, and 1000 lb./hr. for I. The spray and wetted wall experimental figures for a longer column and less abrupt steam entry show mechanical disturbance at large steam flows. Dotted connecting portion joins acetoin and diacetyl sections.

steam flow must be due to mechanical disruption of the liquid films or drops by the steam causing a constantly diminishing value for $(HTU)_l$. In both these cases steam entry was through the side of the wall and not calmed. It is instructive to plot the results obtained for the experimental column (in Part IV), where diacetyl or acetoin was stripped from water in a spray column and a wetted wall column. The curves are flat initially, as would correspond to a constant or nearly constant number of transfer units, but as the steam flow is increased there is a more rapid reduction in the rate of diacetyl removal, although the effect is larger for the shorter columns. It should be noted that acetoin is much less affected by liquid side resistance than diacetyl due to the smaller value of the stripping factor.

Any column (particularly a short column) which shows the effect of mechanical disturbance of the liquid over a wide range of steam flows can have a very small intercept on a $(HTU)_L$ versus I/F plot and can in fact give a negative intercept. The slope of the line is also affected, and care must be taken to appreciate these cases when calculating heights of transfer units for the liquid side and the steam side. The spread of the experi-

mental points (in fig. 5 of Part IV) near the *HTU* axis is to a large extent due to mechanical disturbance of the liquid. The relative effects of steam-flow rates on the steam-side coefficient and the liquid side coefficient can be determined by carrying out experiments using first acetoin then diacetyl.

A continuous counterflow column can be connected to a mixing type plant as shown in fig. 4 of Part II, in fact the experimental points of curve I of Fig. 6 are taken from the results obtained from an experimental unit of this type, the results for cream being very much the same.

A counterflow column must be of adequate size to reduce taint levels to a value comparable with that for single contact stripping, and with suitable design the previously mentioned combination of a counterflow column with a mixing unit can give a useful stripping characteristic.

Table 7 has been calculated for typical cream-flow conditions in a manner similar to the examples given previously. The symbol '2–1 counterflow' means that a counterflow connexion pipe has been added conveying steam from the second vessel to the first. The first vessel to receive the cream is always number 1. Similarly, '3–2–1 counterflow' describes a flow of steam from vessel 3 to vessel 2 and then to vessel 1, which exhausts the steam to a condenser or heat exchanger.

Table 7. *Calculated steam requirements of some deodorizing plants*

(For hypothetical taint of $m=10$.)

Type of plant	Cream preheat by waste heat (° F.)	Residual taint as % of original	Cream flow (G.P.H.)	Total steam flow (lb./hr.)	Lb. steam per gallon of cream
Three-leg Vacreator	145	10	1250	4080	3·66
Five-leg Vacreator	145	10	1700	5240	3·08
Split-flow Vacreator (through 1 Solo to 2 Tandems)	145	10	1500	5640	3·75
Three-leg Vacreator with 2–1 counterflow	145	10	1250	2850	2·28
Five-leg Vacreator					
with 2–1 counterflow	145	10	1700	4240	2·5
3–2 counterflow	145	10	1700	4240	2·5
3–1 counterflow	145	10	1700	3800	2·24
3–2–1 counterflow	145	10	1700	2930	1·72
3–2–1 counterflow	105	10	1700	3180	1·88
3-Vessel continuous counterflow	145	10	900	2480	2·75
Tandem Vacreator	145	10	500	3180	6·35
Tandem Vacreator	145	12·5	600	2940	4·90
Tandem Vacreator	145	25	800	1720	2·15
Tandem Vacreator added mixing section with 2–1 counterflow	145	25	800	1000	1·25

The conditions represented by a Tandem Vacreator reducing the hypothetical taint concentration to 12·5% of the initial value appears to represent the limiting use of a Tandem Vacreator under New Zealand conditions, although a 10% taint level could be attained by a further reduction in cream flow. The figures for the 25% level give a better indication of New Zealand practice because Tandem Vacreators only are often used where taint intensities are not great.

In Table 7, figures are given for the steam requirements for a commercial size 3-vessel continuous counterflow system, but in practice a high degree of heat recovery can reduce

the steam consumption to 2·35 lb./gal. If larger vessels were used the same steam consumption could be obtained with two vessels or even a single vessel. Alternatively, and preferably, if the design of a vessel could be improved so that each counterflow vessel had 4 transfer units, adequate and efficient deodorization could be obtained within a single vessel.

The present limitation of one Transfer Unit per vessel, and the consequent need for three vessels, gives rise to increased cost and space requirements and is a problem of considerable importance. Since most of the resistance to the transfer of taint is in the liquid side, any means by which (*a*) the interfacial area of steam-liquid contact can be increased, or (*b*) the turbulence of the liquid can be increased, will result in a greater number of transfer units per vessel. Obviously, the standard bubble caps and various types of packing would improve the rate of taint-transfer, but their use could cause difficulties due to foaming, unacceptable time of contact, and, of course, difficulties in cleaning of the plant. Apart from suitable sanitary packing, or other means of increasing the internal surface area, the liquid film could be agitated, either mechanically or by means of the steam flowing at sufficient velocity.

The beneficial effect of agitation of a liquid body on mass or heat transfer rates is well known in chemical engineering practice, and possibly it would be simpler to increase the number of transfer units by agitation of the cream film instead of extending the area of contact of the steam and cream.

FLOW CIRCUITS FOR STEAM AND CREAM

Cream deodorizing equipment can be classified under four headings:

(*a*) Continuous counterflow (Part IV).
(*b*) Single or multi-stage co-current mixing (Part I).
(*c*) Generated steam (from boiling cream) (Part I).
(*d*) Vessels of types (*b*) and (*c*) with suitable counterflow connexions for steam (Part II).

In the type (*a*) or continuous counterflow class the only commercial example known to the present author is the recently introduced A.P.V. system (1). Type (*b*) or co-current mixing has been used by a number of manufacturers, very often in conjunction with type (*c*) equipment. The Vacreator is perhaps the best known equipment, but Australian, American and European designs are also in use. Very often a plant is designed to remove taint by flash steam (formed by a heated dairy fluid flowing into a vessel held at some suitable degree of vacuum) but provision is made sometimes to allow extra steam to be injected if the flash-steam release is insufficient to accomplish satisfactory deodorization. Type (*c*) is a special case of type (*b*) and the same fundamental formulae can be used for calculations of either type. The recent introduction of the class (*d*) principle can be widely applied but at present has been used only with Vacreator equipment. The increasing demands for a high standard of deodorizer performance, the high cost and the better understanding of deodorizing equipment should encourage designers to place improved plant on the market. To this end, several different flow circuits are put forward which would be useful in the design of deodorizers for the dairy industry.

Multiple contact counterflow with steam compression

The development of multiple contact deodorizing vessels with counterflow connexions has been based on the flow circuit given in fig. 1 of Part II in which the pressure in the second vessel is greater than the pressure in the first vessel, an interstage cream pump being required to move the cream. A somewhat similar method is shown in fig. 7 where vessel 2 is at a lower pressure than vessel 1 due to the pressure difference caused by the

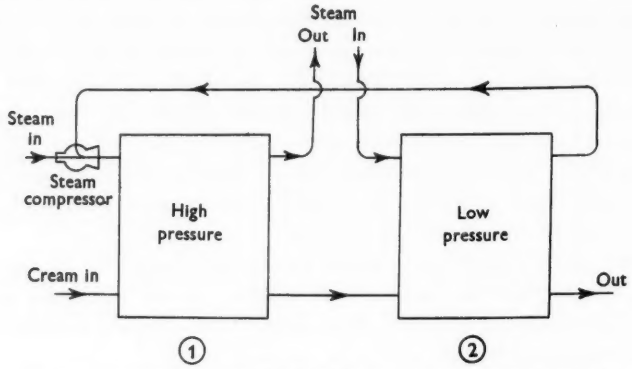

Fig. 7. Double-contact counterflow system. Modified by use of steam jet compressor to return steam.

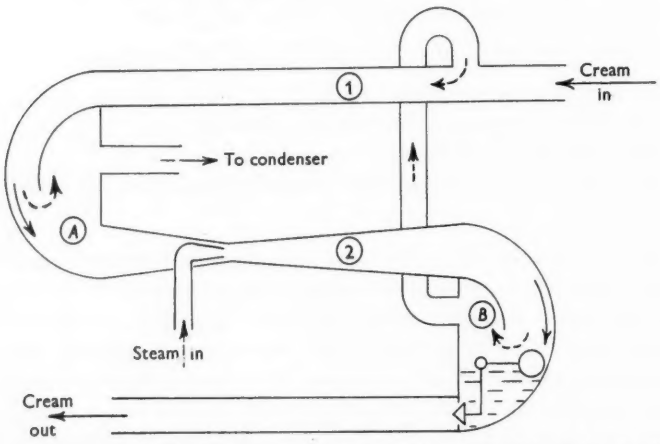

Fig. 8. Diagram of a double-contact counterflow deodorizing unit of simple construction.

steam jet compressor. This method does not require an interstage pump, but has a decreased efficiency because the steam required to operate the compressor is used in one vessel only . Obviously the pressure difference between vessels should be kept as low as possible in order that the steam required for thermal compression be kept to a minimum; but there must be sufficient pressure difference to cause the cream to flow from one vessel to another.

A useful property of the flow system of Fig. 7 is the vacuum augmentation produced by the thermal compressor giving rise to a decreased duty on the main vacuum-producing

means. The circuit can be part of a more comprehensive deodorizing system with the second vessel acting as a partial cooling means for the outgoing cream. Either liquid or vapour pressure can sometimes be used as a pumping medium as, for example, in Fig. 8, where the steam injection section acts as a means for producing a pressure difference.

In Fig. 8, vessel 1 is a length of pipe; vessel 2 is a venturi-shaped length of pipe which causes pressure at separator *B* to be greater than at separator *A* and so allows the partly used steam from the lower section to flow up the interconnexion to be used in the top section before rejection at point *A*. The stripping action is similar to that of a pair of bubble-cap plates, and such units could be used either as deodorizers or to augment existing plant. A 40-G.P.H. pilot plant which was built by the author performed satisfactorily.

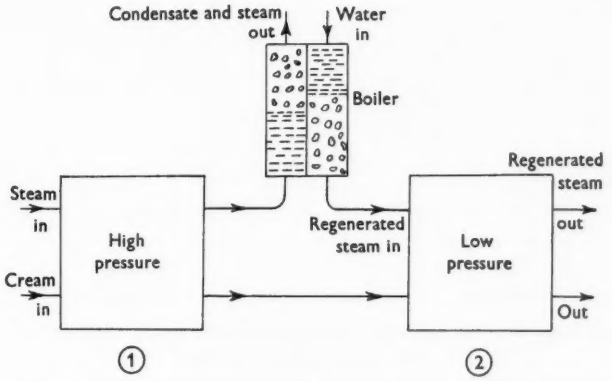

Fig. 9. A steam economy technique. Steam from one section is used to generate steam for a further section.

Regenerated steam

Fig. 9 shows a technique which has been experimentally used by the author. Waste heat from one vessel is used in a calorifier or boiler to produce steam to be used in a second vessel. The feed water should preferably be preheated by waste heat from some later stage in the system, and the general practicability of the circuit depends on the design of the regeneration boiler. The size of the condenser-boiler heat exchanger can be reduced by combining several of the techniques presented here, for instance low-pressure steam from a regenerator can be upgraded in pressure by a steam compressor and used in a multiple contact counterflow circuit with the exhaust steam supplying the heat for the regenerator.

Divided steam flow

When a number of vessels for contacting steam with cream are connected in counterflow the overall vacuum difference from the first vessel to the last vessel can be excessive if the pressure loss per stage is not kept to a low figure. Since the usual condition in the first vessel of a cream treatment plant is about 0 in. Hg, a large difference in vacuum will result in a condenser operating at a vacuum higher than normal. There are some disadvantages in the use of equipment with vessels operating at large differences in vacuum: (*a*) the vacuum-producing apparatus must be suitably increased in size, (*b*) more steam or

water is used to produce the vacuum level, (*c*) the plant is over-sensitive to air leaks or gassy cream, and (*d*) deodorization efficiency can sometimes be reduced by successive condensation of taint-laden steam and can reach a point where the final vessel is a hindrance rather than a help. A plant with four vessels can be arranged, as shown in Fig. 10. The effect of dividing the steam flow through a pair of vessels is to increase the efficiency, this result being due to double-contact action replacing single-contact action. The efficiency must be less than that for four vessels using counterflow steam connexions, and it is not difficult to show that the efficiency of the arrangement in Fig. 10 approximates to the efficiency of a triple-contact counterflow system.

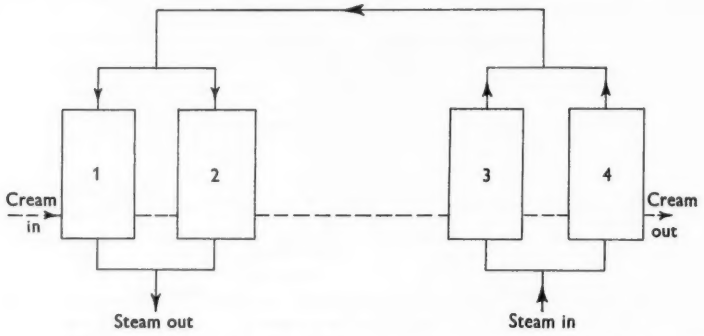

Fig. 10. A divided steam flow technique. Efficiency is greater than a double-contact system and the pressure difference is less.

Often it is possible to change existing equipment to one or more of the flow circuits given above or variations of the flow circuits can be used. Fig. 11 shows how the pasteurizing section of a Vacreator was converted for experimental purposes to a double-contact vessel by the divided-flow technique. Steam is allowed to enter at a point near the bottom of the pasteurizing section (the original steam inlet now being used as an outlet), the spray portion of the chamber thus being converted into a small continuous counterflow column. The initial experiments on this system have been made with a separate condenser to remove the steam issuing from outlet *A*, but it is quite possible to lead the steam from the top portion through a throttle ball or valve to the separating chamber, each Solo unit of a Vacreator would then be equivalent to two vessels.

The points of curve *H* of Fig. 6 were taken from some of the results of experiments using a small Vacreator in this fashion. Usually one of the vessels in a double-contact system must give at least 0·5 of a transfer unit for there to be a gain over a single-contact system and therefore it does not always follow that a divided steam flow system increases stripping efficiency. In the case of the experiments on the small Vacreator, increased efficiencies were most apparent at high steam flows, where mechanical action on the liquid gave rise to an increased number of transfer units.

The final test of any cream deodorizing plant must be the grading score of butter made from the treated cream. In the experimental results to date there is no evidence that the divided steam flow technique affects butter quality, although in the Vacreator conversion, as shown in Fig. 11, there was evidence in some experiments that the change of steam

inlet caused a slight reduction in grading score even when outlet *A* was closed, although repeat experiments did not give the same result. This emphasizes that small changes in plant design may cause changes in cream quality in some cases but not in others.

LIMITATIONS OF EFFICIENCY

For each class of deodorizing plant there is a limiting theoretical efficiency, e.g. the maximum efficiency for multiple contact operation is $x_0/x_1 = e^{-F}$ which can be obtained by batch operation (4). The 100% saturation curve (Figs. 2 or 3) is the limiting efficiency

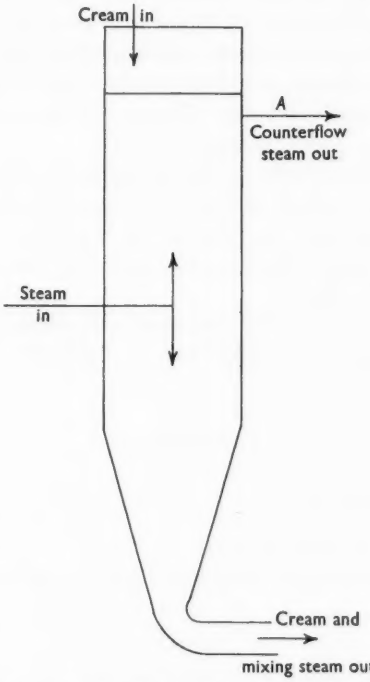

Fig. 11. The divided steam-flow method applied experimentally in a Vacreator vessel.

for counterflow operation. As an example take the case where $x_1 = 100$ in Fig. 4, $m = 10$ and $x_0 = 10$. The solute concentration in the outgoing steam cannot exceed $10 \times 100 = 1000$ and since the solute concentration must be $(100 - 10)/(V/L)$, therefore V/L cannot be less than 0·09 if a value of $x_0/x_1 = 0{\cdot}1$ is to be maintained. If the outgoing steam can be usefully employed for the heating of water, cream, etc., then the heat efficiency can be higher than the above figures would suggest. In general, any arrangement whereby steam for heating can be used first for deodorizing will reduce steam costs in butter manufacture.

Limitations of efficiency are often encountered in practical plant design for other reasons. Where the steam flow is used to carry the cream through the plant, sufficient steam must be used to transport the cream and in general it is better to design deodorizing

plant so that steam is not depended upon for this function. The divided steam-flow principle reduces the rate of steam flow through each vessel and particular care must be taken when applying this principle to Vacreators or similar plant where cream is moved by the action of flowing steam.

SUMMARY

A comparison is made of the stripping characteristics of the types of plant studied in previous papers of the series. The effects of alterations to the type of plant, the number of vessels used or a change in processing conditions on the volatile flavour components are illustrated by calculations for solutes having equilibrium coefficients m of 2, 10 and 50. For design purposes a hypothetical taint of $m=10$ is used, the final taint concentration being set at 10% (of initial) for high-taint pastures and 25% for low-taint areas. Using the above hypothetical conditions, calculated steam-demand figures are shown to agree with known typical steam-demand values. The use of a flash cooling vessel is examined and shown to effect a useful steam saving.

The effect of steam velocity on the mechanical break up of the liquid in a counterflow spray column is shown; an increase in steam flow increased the liquid side disturbance and thus reduced the liquid side resistance giving a greater rate of taint transfer, the characteristic thus more nearly following a line of constant saturation than a line of constant number of transfer units.

A number of flow circuits for deodorizing plant are described. The general conditions limiting the continued reduction in steam flow are discussed.

NOMENCLATURE

e = 2·718.

F = stripping factor $= mV/L$.

G = vapour flow into a vessel (lb./hr.).

HTU = height of a transfer unit, subscripts l and g refer to liquid film and gas film HTU's.

L = liquid flow (lb./hr.).

m = equilibrium constant, where $y=mx$ for distribution of a taint between liquid and vapour.

p.p.m. = parts per million.

Q = ratio $\dfrac{\text{liquid flowing out from a vessel (lb./hr.)}}{\text{liquid flowing into that vessel (lb./hr.)}}$.

S = condensation (lb./hr.).

V = vapour flow out of a vessel (lb./hr.).

x = concentration of a taint in a liquid (lb./lb.). x_1 at entrance to a vessel; x_0 at exit from a vessel; x_a, x_b, x_c at various stages in a multistage process.

y = concentration of a taint in a vapour (lb./lb.). y_1 at entrance to a vessel; y_0 at exit from a vessel.

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619.2

REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION E. DISEASES OF DAIRY CATTLE

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I. MASTITIS

A. *Treatment and control*

The Technical Committee on Mastitis of the Agricultural Research Council has published a method for the eradication of *Streptococcus agalactiae* from dairy herds (1). It is pointed out that this organism has now been eradicated from a large number of herds in Great Britain, and with its removal it has become easier to devise methods of control of other forms of mastitis. Before describing the method a number of points referring to the organism are given: (1) *Str. agalactiae* is, apart from a few notable exceptions, an obligatory parasite of the udder; (2) it is sensitive to penicillin; (3) the route of infection is by way of the teat canal and spread occurs during milking; (4) there is no immunity to reinfection; (5) penicillin treatment will remove infection from more than 90% of infected cows, but, due to the nature of the lesions in some cows, it will not remove it from 100%; (6) it is necessary to supplement the action of penicillin by disinfection of other sites such as the hands of the milkers; (7) teat sores should be treated before eradication is attempted. The method of eradication is given under four main headings: (1) A preliminary examination of the whole herd is made. For this purpose the milk samples may be collected by the farmer himself. (2) All infected and dry cows are treated in such a way as to maintain a sufficient concentration of penicillin in a quarter for 6 days, i.e. two doses of procaine penicillin in mineral oil 100,000 units with a 3-day interval. At the same time attention must be given to milkers' hands and cows' teats. (3) Post-treatment samples are drawn directly on to selective medium and should be taken 6 weeks after treatment, then at 6-monthly intervals. If a herd is free from infection at two 6-monthly tests, sampling at yearly intervals is sufficient. (4) Any cow found to be infected at post-treatment sampling should be treated and milked last. The case of an animal resistant to two treatments will have to be considered individually. To prevent reinfection of the herd any purchased cow must be treated, and for 14 days the hands of any new milker must be disinfected with disinfectant cream before milking. Discussing mastitis in Denmark, Bendixen (2) mentions the fact that eradication of *Str. agalactiae* would still leave the problem presented by other forms of mastitis. It is his opinion that in the control of mastitis the state of buildings is important in that shelter, quiet and adequate warmth must be provided as well as conditions which will prevent teat injuries. In addition, if the milking hygiene is not

* The years covered by this review are mainly 1954 and 1955.

good it will counteract any control measures. In view of the fact that when *Str. agalactiae* has been eliminated from a herd, mastitis caused by other organisms is found to be a problem, Maddy(3) suggests that *Str. agalactiae* should be considered as normal udder flora, and that it might be better not to try to eliminate it, but by careful maintenance of the herd to prevent predisposing causes of mastitis and thus keep infection at a low level. He goes as far as to suggest that a new approach to the problem of mastitis might be of value, that is, the injection of attenuated organisms into the udder of healthy animals to establish a bacterial flora which might prevent the establishment of more harmful organisms.

Discussing the present position Wilson(4) reports that with the introduction of penicillin there was a significant reduction in Great Britain of the number of cases of clinical mastitis, but that this improvement has not been maintained. He suggests that mastitis, as a herd problem, should be considered under three headings: contagious mastitis, 'a-specific mastitis' and acute sporadic mastitis. In his classification contagious mastitis is a type that is spread from cow to cow by hands, cloths and teat clusters and that is, as a rule, subclinical. The organism responsible is generally *Str. agalactiae*, but staphylococci, *Str. dysgalactiae* and *Str. uberis* may be concerned. Eradication is based on good hygiene and bacteriological examination of milk samples and treatment of infected cows. Included under the heading of 'a-specific mastitis' are those cases in a herd where mastitis appears in clinical form, but where the organisms recovered are variable. The spread is not contagious but is believed to be due to some other factor such as lowered resistance in the animal. Acute sporadic mastitis includes all infections which produce systemic effects as well as inflammation of the udder. In this type the route of infection is problematical. The condition is usually confined to isolated cows and the organisms responsible are *Str. dysgalactiae*, *Corynebacterium pyogenes*, *Staphylococcus pyogenes*, Group C streptococci, *Bacterium coli* or *Pseudomonas pyocyanea*. The majority of these organisms can have a saprophytic existence and only develop a pathogenic role when conditions are favourable. While in 'a-specific mastitis' and acute sporadic mastitis treatment of established cases is essential, prophylactic measures based on good hygiene and milking technique are advised. A new compound, 'Hibitane', has been described by Davis, Francis, Martin, Rose & Swain(5), which is claimed to be bacteriocidal to Gram-positive and Gram-negative organisms. Wilson(6) has described successful trials in the prevention of spread of mastitis by udder cloths, hands and teat clusters using this compound. At the strength used, however, it was estimated that the cost would be too high for general use and tests with higher dilutions are being made.

In the previous review of this series(7) reference was made to the use of oxytocin, the hormone responsible for milk ejection, in mastitis therapy. Carefully controlled experiments have since been done at Compton by Smith & Pattison in both goats(8) and cows(9) on the effect of this substance on experimentally produced mastitis. It was found that in goats the use of oxytocin, either alone or in conjunction with penicillin had no effect on the course of experimental mastitis produced by *Str. agalactiae*. In the work on cows eight animals were each experimentally infected with *Str. agalactiae* in one quarter, and each of the quarters so treated developed a clinical mastitis that could not be distinguished from the naturally occurring disease as recognized by bacterial count, neutrophyl count, Whiteside test, milk sediment volume, appearance of the milk and the body temperature of the animal. The animals were paired and one of each pair treated with oxytocin. This treatment caused a spectacular let-down of milk, but analyses of variance of the results for the periods before and after the administration of oxytocin failed to reveal a statistically significant difference between the two groups. Treatment with penicillin gave a bacteriological cure in all eight quarters and no adjuvant action of the oxytocin was demonstrated. The authors point out that in the original work it was presumed that the spectacular action of oxytocin in removing clots and debris from a quarter was bound to be beneficial, and also that the conclusions were based on the purely subjective criteria of a return to normal appearance of what was previously abnormal milk. The team working on experimental mastitis at Compton have laid down strict criteria as to what constitutes typical clinical mastitis caused by *Str. agalactiae* and amongst these the importance of the body temperature of the animal as

an indication of repeated crises is stressed. Their ability to reproduce the disease experimentally puts these workers in an ideal position to test the efficiency of methods of treatment. Conflicting reports on neomycin as a treatment for mastitis were given in the previous reviews of this series(7). More recently, Aynsley & Hughes(10) have found that though mastitis organisms are very sensitive to neomycin in Difco tryptose broth, when grown in skim milk a concentration of more than 40 units/ml. is required to inhibit them. They also found that 24 hr. after infusion of 500–1000 mg. of the drug into a quarter of the udder the level was well below that figure. Only fifteen of forty quarters infected with *St. pyogenes*, *Str. uberis*, *Str. dysgalactiae* or *C. pyogenes* were cleared of infection by intramammary infusion of neomycin. Because neomycin sulphate and polymyxin B sulphate showed synergistic action *in vitro* Christian, Harris & Barr(11) made a trial on forty-two infected quarters diagnosed by means of the strip cup followed by plating of the milk. A single treatment with 100 mg. neomycin and 100,000 units polymyxin B was successful in clearing infection from thirty out of thirty-four quarters infected with *Str. agalactiae*. A single treatment also cleared infection from quarters infected with staphylococci, *B. coli* and *P. aeruginosa*. Vigue(12) has compared the relative efficiency of neomycin sulphate combined with penicillin or bacitracin or with both, in the treatment of mastitis in a herd. The two combinations containing penicillin gave the best results. Konde & Attleberger(13) treated seventy-seven quarters with six different combinations of drugs and found that three combinations containing neomycin were 100% successful in clearing infection. It should be noted that two of these combinations also contained penicillin.

The effect of antibiotic treatment on the cell content of the milk is reported by Blackburn(14), who refers to subclinical mastitis, associated with coagulase positive staphylococci and haemolytic streptococci, and diagnosed as mastitis by means of the differential cell count. Antibiotic treatment eliminated organisms from 61% of staphylococcal and from 80% of streptococcal udder infections. In 84% of quarters from which organisms were eliminated the cell counts of the milk were reduced, about half of them to normal and about one-third to late-lactation type of cell count in which the majority of the cells are epithelial cells. The remainder became reinfected before there was time for the cell counts to return to normal. It was found that it may take as long as 5 weeks for the cell counts to return to normal (less than 70,000 cells/ml.) after elimination of mastitis organisms, but in quarters in which the cell count eventually fell there was a drop in the percentage of polymorphs in the milk from more than 60% to less than 30% 1 week after treatment. The term polymorph is used synonymously with the terms neutrophil and heterophil. Treatment of staphylococcal infection in thirty-seven naturally infected cows is reported by Edwards(15). The animals were taken to an isolation unit and subjected to six pre-treatment tests in a 2-week period and six post-treatment examinations at weekly intervals. The following antibiotics were used: aureomycin, penicillin, streptomycin and a combination of the last two. Aureomycin caused a slight temporary decrease in milk yield with clotting and discoloration of the milk. The results showed no real superiority of one antibiotic over another and infection was eliminated from thirty-one of the thirty-seven cows. Four of the six cows resistant to treatment had five or more lactations, which tends to show that antibiotic therapy is more successful in young cows than in long-standing infections in old cows. Repeated treatment caused no change in the susceptibility of the organisms or their phage type. After intramammary treatment with oxytetracycline (terramycin), Barnes(16) found that most quarters showed evidence of inflammation as estimated by the catalase and Whiteside tests. Treatment cleared infection from only 25% of quarters infected with staphylococci.

McKay & MacPherson(17) report the successful use of iodized mineral oil in cases of udder infection with streptococci and staphylococci which failed to clear when treated with antibiotics or sulphonamide. They recommend that during the dry period quarters showing pathogenic organisms in the milk or a high cell count should be treated with 500 ml. of the oil. Fincher(18) reports that pseudomonas infection of the udder has shown some response to 20% sodium iodide containing polymixin, neomycin or streptomycin. Subcutaneous or intravenous injection of 5000 units/lb. body weight of penicillin was found by Sadek(19) to give a level in the milk within

the therapeutic range of 0·032 unit/ml. for over 24 hr. even with twice-daily milking. A comparison of vehicles in intramammary therapy of bovine mastitis has been made by Schipper (20). Antibiotics were injected into the udder either in 500 ml. of water or in 7·5 g. of ointment. The animal was then slaughtered and the udder removed and frozen in a hanging position after which assays were made of tissue taken from different levels of the quarter. The figures given tend to show that antibiotic concentrations were greater with water as a vehicle but the great difference in the volumes used makes comparison difficult. Richou & Thieulin (21) stress the disadvantages of chemotherapy in the treatment of bovine mastitis and recommend the use of a mixed vaccine supplemented by antibiotics. Amiot (22) states that he has been able to control *Str. agalactiae* mastitis in cattle by means of an autogenous vaccine. A suffusion method for treating gangrenous mastitis is described by Hawley (23). The method consists of the insertion of pilot needles about 3 in. apart around the border of the quarter; longer needles are inserted through them, and terramycin injected. He claims that this treatment was efficacious in four cases of gangrenous mastitis.

A preliminary report on the intramammary use of hydrocortisone in mastitis is given by Vigue (24). Using 'Masticort', with or without hydrocortisone, in the treatment of 188 quarters he claims reduced convalescent time and a higher percentage of cures in the quarters receiving hydrocortisone. 'Masticort' is a preparation containing penicillin, dihydrostreptomycin, sulphamerazine, sulphathiazole and hydrocortisone. Treatment of acute mastitis by local anaesthesia of the nerves of the mammary gland is reported from U.S.S.R. by Logvinov (25). He claims that in the early stages of mastitis the injection of 150–200 ml. of 0·25–0·5% solution of procaine hydrochloride around the base of the teat of an affected quarter brought about an improvement by increasing the circulation, lessening capillary permeability and inhibiting the release of histamine. Ferrini & Sansoè (26) report that the passage of ultrasonic waves through an inflamed udder had no curative effect on the condition.

Numerous papers concerning *in vitro* tests of antibiotics have been published in the period under review. Studying antibiotic resistant staphylococci in subclinical mastitis, Alford, Lusi & McCrory (27) found that of 310 colonies obtained from 1800 milk samples, less than 1% were resistant to penicillin, aureomycin or dihydrostreptomycin. After a treatment trial of nine infected cows they concluded that the development of strains of staphylococci resistant to antibiotics accounts for only a small percentage of cases of mastitis which fail to respond to antibiotic therapy. Huebner & Scheetz (28) made an examination of thirty-one cultures of *Staph. aureus* isolated from cases of bovine mastitis which had been refractory to treatment. Using the serial-tube dilution method they found that about 70% were sensitive to penicillin, bacitracin or erythromycin. Neither streptomycin nor polymyxin B were effective against any of the strains. A technique using methylene-blue milk to assay levels of aureomycin and terramycin in body fluids, including milk, is described by Schipper & Petersen (29). Hop (30) reports that of 158 strains of bacteria isolated from mastitis in cows, ewes and sows, staphylococci were the most sensitive to chloramphenicol and aureomycin, and coliform organisms were the most sensitive to chloramphenicol. Using a dilution series on milk ranging from 1 in 2 to 1 in a million Florestano & Bahler (31) have tested the activity *in vitro* of a combination of penicillin, dihydrostreptomycin, neomycin and sulphisoxazole against micro-organisms associated with mastitis. Staphylococci were inhibited in all dilutions, and *Str. agalactiae* in all but the highest dilution. Gram-negative organisms grew at levels below 1 in 2000.

Investigating the stability of penicillinase-producing strains of *Staph. aureus*, Fairbrother, Parker & Eaton (32) found that out of 200 apparently resistant cultures, six gave both penicillin-sensitive and penicillin-resistant colonies, although they were of the same phage pattern. Gould (33) reports that penicillin-resistant *Staph. pyogenes* derived *in vitro* from a sensitive strain were of two types. One type produced no penicillinase and was of the same phage type as the parent strain, the other produced penicillinase and was of phage type group III even when the parent strain was of group I or group II. It would appear therefore that the isolation of a resistant organism of different phage group does not necessarily indicate a second infection.

Studying chloramphenicol resistance in *Staph. pyogenes*, Wilson & Ramsey (34) found that the addition of yeast extract appeared to antagonize chloramphenicol, whereas asparagus juice enhanced its action. Pre-incubation of the organism (35) apparently due to the increased rate of metabolic activity caused it to be more resistant to the antibiotic. *In vitro* results presented by Trace & Edds (36) indicate that cobalt enhances the antibacterial effectiveness of penicillin, streptomycin and bacitracin against *Staph. pyogenes*. No benefit was noted when cobalt was combined with terramycin, aureomycin, chloramphenicol or polymixin. Examining the effect of combinations of antibiotics on lysis of *Staph. aureus* by penicillin, Kirby & Burnell (37) found that aureomycin, terramycin and chloramphenicol all cause a marked modification of the lytic action of penicillin on the organism. Alterations in the patterns of lysis appeared to be closely related to a slowing in the rate of killing as compared to that with penicillin alone. Tacking (38) reports that treatment with penicillin may enhance the pathogenicity of penicillinase-producing strains of *Staph. aureus*.

B. *Bacteriology and diagnosis*

Staphylococci. A selective medium, tellurite-glycine agar, for the detection of coagulase-positive staphylococci has been described by Zebovitz, Evans & Niven (39). This medium is stated to be superior to others currently in use. Coagulase-positive staphylococci produce black colonies after 24 hr. at 37° C., whereas other organisms, with the exception of coagulase-negative staphylococci, fail to produce visible growth in this time. Colonies of coagulase-negative staphylococci are grey. All black colonies isolated from this medium proved to be coagulase-positive, and in no instance were coagulase-positive strains isolated from phenol-red-mannitol-salt agar and not from this medium. The value of plasma of various species in the coagulase test for mastitis staphylococci is discussed by Klatt (40), who concludes that human and rabbit plasma give the best results, while plasma from horses and pigs is less satisfactory and that from cattle quite unreliable. He has found a constant correlation between the result of the coagulase test and mannitol fermentation. Klastrup (41) gives an account of the technique of a coagulase test using solid medium. It is reported by Duthie (42) that staphylococci produce two forms of coagulase. One is bound to the cell wall and is responsible for the slide test, whilst the other is liberated as free coagulase in the medium and is responsible for the tube test. In a 3-year period, Chute, Witter & Rountree (43) cultured samples of milk showing a total polymorph count of over a million. Staphylococci which were β-haemolytic on calf blood agar were tested for coagulase, and of 1288 cultures tested, 65·9% were coagulase-positive. They found that the majority of cultures which were β-haemolytic were *Staph. albus*. Deubler (44) found that 70% of the quarters in a herd were shedding staphylococci at some time during a 12-month test period. Of seventy-two haemolytic staphylococci 75% were coagulase-positive.

Price, Neave, Rippon & Williams (45) have described the use of phage typing and penicillin sensitivity tests in studies of staphylococci from bovine mastitis. They found that in heavily infected herds infections were due to more than one phage type. Over the whole study staphylococci with pattern A, which includes the 42 D type regarded as typical of bovine strains, were the commonest, but pattern C, with lysis by 42 B and one or more of the phages 29, 44, 52 or 52 A, was nearly as common. Only thirteen of 104 quarters studied contained two types of staphylococci, and, with one exception, two types were isolated only when abrasions were present at the teat orifice. All of 412 strains of patterns B, C or D were sensitive to penicillin and all of 242 strains of patterns L or M were resistant.

It was noted by Elek & Levy (46) that, when a streak of *Staph. aureus* is grown on nutrient agar at right angles to an antitoxin-soaked filter-paper, distinct antigen-antibody flocculation lines appear. Howard (47) has found that the number of such lines is directly related to the virulence of the organisms for mice, and that the formation of large numbers of lines is associated with the production of higher titres of α-toxin in broth cultures. Elek & Levy (48) report that, because of interaction between haemolysins, quantitative tube titrations of culture filtrates from coagulase

positive staphylococci are unreliable. Sandvik(49) has studied the haemolysis produced by strains of bovine staphylococci on blood-agar media. The type of haemolysis seen with strains producing α- and β-toxin depended on the relative rates of production and diffusion of these toxins in the media concerned. Frequently the action of one toxin inhibited the effect of the other. The haemolysins, fibrolysin and coagulase production of 435 strains of *Staph. pyogenes* have been studied by Lack & Wailling(50), but the results could not be correlated with pathogenicity. A definite correlation between staphylococcal udder infection and serum or whey titres of staphylococcal haemolysin antibody has been found by Lasmanis & Spencer(51). The serum titres were usually much higher than those found in the whey. There was a significant correlation between the antibody titre of the whey and the pH of the milk, suggesting that permeability of the mammary tissue may be partly responsible for the antibody in the milk. Munch-Petersen(52) reports that certain slightly haemolytic diphtheroid bacilli occasionally found in aseptically drawn cow's milk excrete a substance which protects sheep and ox red blood cells from staphylococcal β-toxin, with which, however, it does not combine. The agent is thermolabile, filter-passing and apparently distinct from haemolysin. The organism is probably closely related to, if not identical with, *Corynebacterium flavidum* (Morse). The occurrence of a haemolysin-inhibiting substance produced by non-haemolytic staphylococci which inhibits the *delta* haemolysin of staphylococci is reported by Liu(53). A strain of coagulase-negative staphylococcus which exhibits antihaemolytic properties is reported by Pier(54). The antihaemolytic substance, which is thermolabile, does not appear to be specific against any particular haemolysin but rather makes the red blood cell membrane more resistant to lysis. The exaggerated zone of lysis in the CAMP reaction with *Str. agalactiae* is not evident in blood-agar plates containing the substance. These reports suggest possible drawbacks to the sterilization of the udder by antibiotic treatment.

Streptococci. A selective medium for the isolation of group B streptococci from milk is described by Hauge & Ellingsen(55). To a modified Edwards's medium is added 4–6% β-toxin prepared from a heat-treated culture of a strain of staphylococcus. Group B streptococci give a broad zone of haemolysis on this medium, so also do certain strains of *Str. uberis*, but the latter can be differentiated by their action on the aesculin in the medium. Hansen & Winther(56) report a modification of this medium using a lower concentration of toxin (1–2%). A capillary tube test for the detection of bacteria associated with bovine mastitis has been developed by Jones & Helwig(57). Capillary tubes, 92 mm. long and 0·8 mm. inside diameter, are prepared by introducing into them a 0·045% solution of bromcresol purple and driving off the solvent in an oven. After sterilization the tubes are used by making one end touch a stream of milk drawn from the cow's teat, after which the tubes are inserted into clay which plugs the lower end. After incubation at 37° C. for 18–24 hr. a yellow colour indicates the presence of *Str. agalactiae.* Of 1228 quarters tested only twenty-eight showed disagreement between this method and the Hotis test. A milk-ring test, using haematoxylin-stained antigen, for the detection of agglutinins to *Str. agalactiae* is described by Smith(58). The test detected agglutinins in the milk of five experimentally infected cows and was not inferior to the whey slide-agglutination test. The antigen appeared to be species specific. Type classification by Lancefield's precipitin method of human and bovine group B streptococci isolated in Britain has been carried out by Pattison, Matthews & Maxted(59). Type strains of Stableforth's sixteen types of group B streptococci were examined for the presence of the type-polysaccharide on which Lancefield's classification is based. In five types no type-polysaccharide was detected. Examination of 236 strains isolated in Britain showed that 193 reacted with one or other of Lancefield's type sera. The remaining forty-three, all bovine strains, showed only protein antigens of undetermined importance in systematic classification. They found that all strains belonging to Lancefield's type I a or I b were of human origin. Unsuccessful attempts, including passage through mice and goats, were made by Pattison, Matthews & Howell(60) to demonstrate type-polysaccharide in the above-mentioned forty-three bovine strains. They were found to contain one or other of two non-specific protein antigens, namely R (one of the antigens present in group A type 28) and X, an antigen not hitherto described. It was

concluded that group B streptococci may be classified by using the four type sera Ia, Ib, II and III, plus two additional sera containing the proteins R and X by which those strains that are devoid of type-polysaccharide may be separated into two classes.

Diagnosis. The relative diagnostic efficiency of indirect tests, as judged by the percentage agreement they give with the results obtained by bacteriological examination of the milk, is given by Narayanan & Iya (61) as follows: strip cup, 3·8%; digital palpation, 21·2%; bromthymol blue paper, 32·8%; rennet coagulation, 42·9%; chloride content, 51·2%; resazurin reduction, 67·0%; leucocyte count 80·4%; Hotis test, 91·3%; and microscopic examination of incubated samples for streptococci, 98·4%. Taking the presence of mastitis organisms in the milk as being indicative of mastitis, Blackburn, Laing & Malcolm (62) found that on a percentage basis the total and differential cell counts considered together showed no marked advantage over the total cell count alone in the diagnosis of mastitis except in milk from cows in late lactation. Macadam (63) found that transport by lorry caused a two- to tenfold rise in the cell counts of the milk from twenty-eight of thirty quarters examined. The relative increase in cell counts after the journey was greater in the normal udder and in quarters with little mastitis than in quarters having milk in which the cell count was already high and which showed extensive mastitis on histological examination. For seven quarters, all of which showed acute lesions, the average percentage of polymorphs in the total cell count of the milk rose from 64% before a journey to 80% after it. A positive relationship was found between the cell count and pathological lesions, cell counts of over 1,000,000 cells/ml. being found in nine quarters which showed extensive lesions. The author uses an ingenious scoring system to give in a single figure an assessment of the severity and extent of the lesions found in the udder. Cell counts of platform samples of herd milk have been made by MacLeod, Anderson & Plastridge (64). They found that the incidence of mastitis was highest in July and August and lowest in the period from March to June, whereas the cell count was highest in the period from July to October and lowest in January and February. They conclude that factors other than the number of animals with mastitis in a herd may affect the cell counts of the herd milk. It should be noted that animals which are in late lactation will increase the total cell content of the herd milk. Damude (65) reports on the use of the 'Resazurin Field Test' on milk from 1595 quarters. He found it to detect 87·4% of samples which had a cell count of 500,000/ml. or more. When samples showed a cell count of 900,000/ml. or more there was 92% agreement. Recently, in Italy, Ferrini & Piccotin (66) have confirmed that the casein number of milk from cows with streptococcal mastitis was lower than that of milk from normal cows.

C. *Experimental mastitis*

In the previous review of this series (7) reference was made to the experimental infection of cows with *Str. agalactiae* by the Hadley-Wisconsin swab technique. In a series of papers Murphy & Stuart report further experiments using this method of exposure to infection. Six cows in their first or second lactation were exposed in each quarter of the udder four times at intervals of 5 weeks (67). Intramuscular treatment was used to remove infection in preference to intramammary treatment so as to avoid enlarging the teat sphincter. Two animals were infected at all or most of the exposures; two animals were occasionally infected and two animals withstood infection at all exposures. The animals which did not become infected, however, showed organisms in the milk up to the second to the ninth milking. Exposures were repeated (68) on the two susceptible animals and on the two resistant animals, but all were first protected by means of intramuscular penicillin. Penicillinase was incorporated in the culture medium. The milk from the resistant cows was clear of organisms in from three to five milkings, and from the susceptible cows in from two to three milkings. The authors suggest, therefore, that the teat canals of the susceptible cows did not have a structure that retained the organisms in a non-growing state for a longer time than the teat canals of the resistant cows. A method was developed for collecting milk samples by means of a syringe and needle through the wall of the teat so as to avoid gathering organisms from the teat canal (69). The same four cows were exposed to infection and

it was found that the test organism was never recovered from the milk drawn through the teat wall of the resistant cows, but always from that drawn through the teat wall of the susceptible cows. This showed that, though organisms can be found in the milk sampled via the teat canal for three to five milkings in resistant cows, the organisms were not present in the gland but only in the teat canal. In susceptible cows the organisms were multiplying in the gland. The authors (70) then compared 'milking machine rate' and susceptibility to infection by the swab technique. They found that apart from the two highly susceptible animals of the previous experiments which were in the high-rate group of milkers, there was no correlation between high rate of milking and susceptibility. They suggest two possibilities as to the difference between their findings and those of the well-planned and well-executed study of Dodd & Neave reported in the previous review of this series (7): (1) The swab method might not approximate to natural infection closely enough to bring out the relationship between infectibility and dilatability of the teat canal. (2) There might be a fundamental difference between infection with *Str. agalactiae* and infection with other organisms, in which *Str. agalactiae* infection would not be related to the dilatability of the teat canal, whereas infection with the other organisms is. Using vinyl plastic tubing cut to 4 or 5 cm. lengths (71), the lengths of the teat canals were measured in six animals. The tubes were inserted into the teat canal until the milk ran out and the length inserted noted. It was found necessary to insert the tube three times, the first two to 'ream out' the canal. It was found in these six animals that the length of the teat canal was not related to infectivity.

A valuable experimental study of the control of *Str. agalactiae* infection in dairy cattle using ten sets of homozygous twins has been made by Davidson, Slavin & Stuart (72). One member of each set of twins was put into two groups, A and B, and each group divided into two sections. A 'donor' cow experimentally infected with *Str. agalactiae* was put into each of the four sections. Group A, sections 1 and 2, were housed on one side of a cowshed and group B, sections 3 and 4, on the other. The 'donor' cows in sections 1 and 3 were changed over every other day, as were those of sections 2 and 4. In group A a freshly sterilized set of milking machine equipment was used for each animal at each milking, whereas in group B one machine was used throughout for all the animals in that group. In group B eleven quarters of five cows became infected, and six quarters of three cows showed clinical signs with a 40-fold rise in cell count. Three quarters secreted pus at some stage of the experiment. In group A only two quarters in one animal became infected and only one sample showed clots in the milk. This cow's twin was the most susceptible animal in group B, and both had very slack teat sphincters. It was concluded that, when the milking machine as a source of infection was removed, only one cow became infected. There was ample opportunity for the milker's hands to carry infection as *Str. agalactiae* was isolated from seven out of twenty hand-washings and from seven out of sixty-eight swabs taken from bucket handles. In addition, during the first 3 weeks of the experiment when three 'donor' cows were excreting pus in the milk the organism was isolated from enriched teat swabs taken from eight cows in group A.

An attempt to assess increased resistance of the udder of goats to infection with *Str. dysgalactiae* strain 419, after vaccination with the same strain, has been made by Smith, Pattison & Holman (73). In four of five unvaccinated controls crises occurred on nine occasions, whereas in the vaccinated group of five animals crisis occurred only on one occasion. The authors point out that the uncertainty as to whether goats receiving intramammary challenge will react as maximal or minimal reactors makes precise interpretation of these results difficult, but the experiment seemed to give sufficient evidence to warrant immunity tests being carried out on cows. Howell, Smith, Holman & Pattison (74) report on immunological studies in the cow. A group of twenty-two cows received subcutaneous injections of formolized 18 hr. broth cultures of *Str. agalactiae* strain A 100, and a group of twenty-two controls received similar amounts of formolized broth. All forty-four animals were exposed to a single intramammary test dose of the living organism in one quarter of the udder in doses of 1×10^5, 1×10^7, and 1×10^8 organisms. In the second week after infection the vaccinated animals became free from infection more frequently and had lower neutrophil counts than the controls, and in addition the vaccinated cows tended to show an

increase of milk yield in the second week compared with that in the first week. Five animals which received the lowest test dose were exposed to the same dose 16 months later and four of them still showed a high level of resistance. Howell (75) found that of six cows, examined 13 weeks after exposure to intramammary infection with *Str. agalactiae*, three showed serum inhibitor to *Str. agalactiae* hyaluronidase and five had inhibitor to *Staph. aureus* hyaluronidase. A further 116 cows were tested, and though all were negative for inhibitor to *Str. agalactiae* hyaluronidase, fifty of them had inhibitor to *Staph. aureus* hyaluronidase. Milk samples from 18 cows, whose sera contained *Staph. aureus* hyaluronidase inhibitor, were examined for coagulase-positive staphylococci and only three were persistent carriers. It is considered that the sites of infection of staphylococci in the animal may be elsewhere than in the udder. Howell, Pattison, Holman & Smith (76) report the results of an investigation into the relationship of serum staphylococcal hyaluronidase inhibitor to the infectivity of *Str. agalactiae*. The experiment involved forty cows, the serum of twenty-three of which possessed the inhibitor while the serum of seventeen did not. Each cow was inoculated into the teat sinus with *Str. agalactiae*. A significant difference was obtained with the ability of the inhibitor-positive cows to resist infection as compared with the inhibitor-negative cows. There was also a negative association between cows possessing inhibitor and the occurrence of crises. It is suggested that it is unlikely that such inhibitor is directly concerned with the resistance of some cows to *Str. agalactiae*, but that it occurs in parallel with some other protective factor.

A series of experiments to compare the reactions to intramammary inoculation of *Str. agalactiae* in cows and goats has been made by Howell, Pattison, Holman & Smith (77). In three separate experiments, thirty-five cows were inoculated in one quarter of the udder with one or other of two serologically distinct strains of *Str. agalactiae*, strain S 13 and strain A 100. Some 50 % of the cows resisted infection, whereas almost all the goats were readily infected, but once the disease was established it was apparently similar in the two species. The crisis syndrome noted as occurring in about 60 % of the goats was also seen in about the same percentage of cows successfully infected. To record the crisis syndrome temperatures of the animals were taken every 6 hr. throughout the day and night because the febrile reaction can be of short duration. A transient reaction was noted in some cows inoculated with strain S 13 which was not observed in goats, neither was it observed in any cows inoculated with strain A 100. The host response, as shown by neutrophilic invasion, loss of milk yield and febrile response, was severe in this type of reaction, and was associated with rapid elimination of the organisms from the milk. The authors consider that, as the overall picture so closely resembles an immune reaction, it cannot be considered as valid infection. It would, however, be better to say that mastitis was established, as shown by the criteria mentioned, but the cow, having a high degree of immunity, eliminated the infection. The authors suggest that the systemic disturbance noted at times of crisis and the immune response, indicated, by a rise in serum agglutinins, make it important to consider mastitis as a disease of the cow as a whole, not merely of the mammary gland. They also advise caution in the interpretation of observations made after repeated intramammary inoculations of the same animal.

D. *General*

In a paper on the histopathology of mastitis Helmboldt, Jungherr & Plastridge (78) give an extensive review of the literature of bovine mastitis. They examined 505 quarters taken over a period of 20 years from a herd free from brucellosis. Their findings indicated that mastitis was almost solely an acute inflammation and that chronic mastitis, if defined on the basis of histopathology, was relatively uncommon and existed chiefly as a clinical entity. In every case of mastitis, excepting some cases of coliform mastitis, the heterophil was a prominent feature. The lesions of acute mastitis varied from a microscopic focus involving a lobule or a fraction thereof to macroscopic foci involving a large portion of the quarter. The process may repeat itself on a microscopic scale, striking a few lobules and then forsaking them for others; it may cease altogether or there may be a major catastrophe resulting in great destruction of tissue.

Oliver, Dodd & Neave(79) give a description of a dairy herd, its breed composition and management over a period of 8 years. The incidence of udder infection and mastitis was studied in 530 complete lactations. Using the figures obtained from this herd, Oliver, Dodd, Neave & Bailey(80) found that with advancing lactation there was a decline in the rates of first infection, reinfection and total new infection and also in the incidence of mastitis in infected quarters. In early lactation there was a marked tendency for infected quarters to develop clinical mastitis. It was found also that there was an increase of infection and mastitis with advancing age, but infected older cows were not more liable to develop clinical symptoms than younger cows. There was an indication that the longer a quarter was free from infection the greater were its chances of remaining so. No major seasonal trends were found in the incidence of infection or mastitis, though the incidence was slightly higher in the summer months. Oliver and his colleagues (Oliver, Dodd, Neave & Lee(81); Oliver, Dodd & Neave(82)) have studied udder infections in the dry period. It was found that the taking of samples of secretion from the dry udder for bacteriological examination did not increase the incidence of new infection in the dry period, and it was concluded that if natural sealing of the teat orifice takes place it is of minor importance in preventing infection in the early part of the dry period. Comparing sudden cessation of milking and intermittent milking as methods of drying-off cows, it was found that in quarters of cows not infected at drying-off there was a significantly higher incidence of new infection following sudden cessation of milking than following intermittent milking. Cows with higher yields at drying-off were more prone to new infection in the dry period than cows with lower yields. Disinfection of the teats reduced the incidence of new infections in the dry period.

The effects of vacuum level and milking duration on Guernseys and Holsteins differing with respect to lactation number and udder health have been studied by Mochrie, Hale, Dembiczak, Eaton, Plastridge & Johnson(83) at Storrs Agricultural Experimental Station with reference to the cell counts of the milk, chloride content, milk yield, fat content, changes to the teat and 'fibrosis' of the udder as revealed by palpation. Variations of vacuum level between 10, 13·5 and 17 in. showed no significant differences. Milking for twice the 'normal' duration caused greater weekly increases in the cell counts than milking for a normal time. It was found by workers at Storrs(84), contrary to previous reports, that animals classified as infected with mastitis had slower rates of milk-flow than animals classified as normal.

A strain of *Actinomyces* (*Nocardia*) *asteroides* recovered from a case of mastitis in a cow is described by Munch-Petersen(85). When the organism was injected into the teat canal of normal quarters it produced thick-walled nodular abscesses 1–10 mm. in diameter, containing green-yellow viscous pus of earthy odour. As grittiness of the pus was absent it was assumed that the club formation of the typical actinomycotic lesion was absent. Histological examination of the tissue does not appear to have been carried out to confirm this. Galli(86) reports two outbreaks of 'mycotic' mastitis in cattle, both following intramammary injection of penicillin. One outbreak, benign in type, was due to *Candida pelliculosa* and the nine infected cattle recovered after treatment. A more serious outbreak in which twenty-six cattle were infected so badly that they had to be slaughtered was caused by *Debaromyces neoformans*. The author suggested that contaminated distilled water used in the penicillin treatment was the source of the infection. Reporting on pseudomonas mastitis Tucker(87) states that, as a rule, large numbers of bacteria are found in the milk before symptoms arise. Deubler(88) found that in a quarter with pseudomonas mastitis the secretion may vary from a straw-coloured watery fluid to thick purulent material. The duration of an attack is not usually more than 2–3 days, but in both treated and untreated quarters the infection may become active again. The author reported the isolation of many strains of pseudomonas which do not produce chloroform-soluble pigment. An outbreak of udder infection by a streptococcus of Lancefield's group G is reported by Barnum & Fuller(89). Although the organism appeared to establish itself in many udders an increase in cell counts was not a constant feature. Barnum(90) has recorded a severe outbreak of mastitis associated with *Pasteurella multicida*, and Barnes(91) four cases of mastitis caused by *Klebsiella pneumoniae*.

In 1954 an investigation was made into summer mastitis in Britain by Weaver and other

final-year Students of the Royal (Dick) School of Veterinary Studies(92) whilst 'seeing practice' with veterinary surgeons. Milk samples were collected during July, August and September, and sent to the Bacteriology Department of the School for examination. The quarters were selected for sampling on the basis of acute onset of mastitis with systemic disturbance; the quarters, firm and enlarged, and the secretion, containing pus, being difficult to withdraw and of obnoxious odour. Of 116 samples taken, sixty-three showed *Corynebacterium pyogenes* either alone or mixed with staphylococci, streptococci or coliform organisms, and fifty-three were negative for this organism. No correlation was found between the presence of *C. pyogenes* and humid weather conditions. There appears to have been some misunderstanding as to what is generally understood by the term 'summer mastitis' in that some samples were taken from cows in lactation. Pulsford(93) reports that mastitis associated with *Str. agalactiae* is widespread in South Australia. It has been found that in some districts veterinary surgeons appear to have exerted a marked influence on the control of the disease, whereas in other areas free availability of penicillin to farms in the absence of supervision has had little effect.

Stimulation of *Str. agalactiae* by raw milk possibly due to traces of sulphur compounds is reported by Auclair(94). Milk from quarters with mastitis has a greater effect than that from healthy quarters. The same author(95) has found that the milk of cows susceptible to mastitis has about the same amounts of the two inhibitory substances, Lactenin 1 and Lactenin 2, as milk from resistant cows, and Krüger(96) found that there was no difference *in vitro* in the inhibitory activity against *Str. agalactiae* of samples of milk from susceptible and resistant cows. Two methods of estimating the resistance of milk to the growth of *Str. agalactiae* have been compared by Pounden & Frank(97). The first is the length of time required following inoculation for multiplication to be detected and the second is the amount of acid produced. They concluded that the two methods were not comparable as measures of resistance of milk to the growth of *Str. agalactiae*.

II. CONTAGIOUS ABORTION

A. *Diagnosis*

In a previous review of this series(98) it was reported that in fluid taken from the uterine mucosa of cows positive brucella agglutination titres of 1 in 5 to 1 in 1280 had been demonstrated, and in a number of cases the blood titre was less than that of the uterine fluid and was sometimes negative. More recently, evidence of the local production of brucella antigens in the uterus and vagina has been presented by Kerr(99). He found that circulating antibodies produced by intramuscular injection of live *Brucella abortus*, strain 19, did not pass into the lumen of the uterus or vagina except during the calving period. On the other hand, the high titres obtained from the vagina or uterus which followed instillation of dead *Br. abortus* into the uterus did not pass into the circulation. He considers that the finding of vaginal antibodies to *Br. abortus* in a herd indicates that a virulent strain is present and that the finding of such antibodies should be a useful aid to diagnosis. Romváry(100) examined by agglutination and complement-fixation techniques the vaginal secretion of 270 cows from four infected herds and from one herd free from infection. Milk, whey and serum from the same animals were also examined. Cows with milk, whey and serum positive to the tests and whose vaginal secretions taken before and after parturition were negative, did not abort. For cows that aborted, brucella antibodies were demonstrated both before and after abortion in the vaginal mucus. These two reports point to the importance of the presence of brucella antibodies in the vaginal secretion of cows in detecting animals that are likely to abort. They also stress the danger of such animals as a source of infection to others.

Using isolated cattle of known brucella history, Ogonowski & McDiarmid(101) compared the milk-ring test, the plate test and the whey-agglutination test. Provided that a borderline reaction to the milk-ring test was accepted as negative all three tests were negative to the milk from uninfected cows. Only a small proportion of the tests on the milk of infected cattle were

completely negative. They consider that the plate test is more accurate than the other two tests, as well as being easier to perform. There was apparently little interference with the tests by vaccination with strain 19 provided that the vaccination was done before breeding age. The authors point out that after 10 years of vaccination in Great Britain the incidence of brucellosis is now reduced to a level where eradication can be contemplated, and in these circumstances the plate test of milk samples would be a good method to test for agglutinins. They add that it might be necessary to restrict vaccination to calves of 4–9 months of age because vaccination at a later age may give rise to agglutinins. Negative reactions to the milk-ring test for a period of 2 months after parturition were obtained by Ortenzi & Valente(102) on two groups of cattle. The animals in one group were vaccinated with strain 19 between the ages of 7 and 8 months and those of the other group were vaccinated at 6–10 months and revaccinated during the first month of pregnancy. Tallman & Herman(103), however, report that most cows vaccinated as adults give positive reactions to the milk-ring test. They claim that non-specific ring-test reactions in milk brought about by ageing of the sample under refrigeration and by the use of certain antigen preparations can be overcome by heat treatment of the samples before testing, and further that such treatment does not appreciably alter the specificity of the test for positive samples. The effect on the tube-agglutination test of incubation at 56° C. instead of 37° C. is reported by Morse, Schneider & McNutt(104). There was a decrease in the titres of sera from many suspect and low-titre reactor cattle (below 1:400), but rarely a reduction in titres of sera that reacted at 1:800 and over. Reduction in titres was more pronounced with sera from vaccinated than from unvaccinated animals. Evidence is presented to show that such incubation inactivates the non-specific agglutinins more than the specific agglutinins. A survey of fifty-four herds, comprising 3570 cows, has been made by Cameron & Kendrick(105). The results indicated that serum titres of up to 1:200 or at least a maximum of 1:100 are not indicative of localization of *Br. abortus* infection in the udder provided that there are no cows with higher titres in the herd. Some animals in herds free from brucellosis may give a weak positive (1:100) or a doubtful (1:50) reaction to the agglutination test. Orlov(106) is of the opinion that a complement-fixation test should be done in these doubtful cases, and both tests repeated in 2–3 weeks, the animal being isolated meantime. If the titre does not rise and the complement-fixation test is still negative the animal is not infected. A conglutination complement-fixation test is described by Engelhard & Carlisle(107), which can be used for the detection of non-haemolytic complement fixing antibodies in brucella-infected bovine animals. A surface fixation method for detecting brucella agglutinins in serum is described by Castaneda(108). Small circles of stained antigen are printed on filter-paper, where they remain sensitive for several months. A drop of unknown serum is placed over a spot of antigen and positive and negative control sera are placed on neighbouring spots. The reaction is developed by capillary adsorption of isotonic saline in a manner similar to paper chromatography. In a negative test the antigen is removed from the spot, whereas in a strong positive reaction it remains fixed to the spot. Intermediate degrees of intensity may be estimated by comparison with the controls. The test appears to have remarkable specificity as judged by blood cultures.

Studying cross-agglutination between *Vibrio foetus* and *Br. abortus*, Kiggins, Plastridge, Williams & Easterbrooks(109) conclude that *V. foetus* infection does not interfere with the agglutination test for *Br. abortus*, but *Br. abortus* infection may interfere with the *V. foetus* agglutination test if the antigen is made from strains which possess antigenic compounds shared by *Br. abortus*. Stained brucella antigen was tested by Anczykowski & Murat(110) on 3487 serum samples known to be positive, suspect or negative for agglutinins. The results did not differ from those obtained with unstained antigen, but the stain helped in reading the test. Farina & Biggi(111) describe a modification of the agglutination test in which the serum is diluted with cow's whole milk. One drop of antigen stained with tetrazolium is added to each tube and readings are made after 1 hr. incubation at 37° C. The use of 2:3:5-triphenyltetrazolium chloride in a loop test and also in a rapid slide test using whole blood is described by Elek & Vizy(112). Approximately 90% of samples positive to the tube-agglutination test were positive also to

these two tests. It is pointed out by Clapp(113) that, though agreement of positive results of blood tests and the milk-ring test may be only in the region of 80%, for survey purposes the test is required to find only the percentage of infected cows. In a series of 449 cows 25·8% were positive to the blood test and 26·8 positive to the ring test. Clapp is of the opinion therefore that the milk-ring test is of approximately equal value to the blood test for detecting the number of infected cows.

Discussing the international standard for anti-*Brucella abortus* serum, Stableforth(114) states that the international standard or a national standard of an equivalent potency ensure comparability of titres in different countries by different methods. A serum standard of reference was used for examination of agglutinating suspensions prepared in laboratories in trials on eradication of brucellosis from dairy herds in Great Britain which began in 1937. This standard was adopted in 1937 by The Office Internationale des Epizootics (O.I.E.) which made recommendations for its use internationally. These recommendations were revised by O.I.E. in 1948 and by the Joint FAO/WHO Expert Panel on Brucellosis in 1950 and 1952. A new batch equivalent in potency to the original standard was established by a WHO Expert Committee on Biological Standardization in 1952 as the International Standard for Anti-*Br. abortus* serum.

B. *Bacteriology*

An improved method for the cultivation of brucella from blood is described by Braun & Kelsh(115). Laked blood samples are passed through membrane filters and the membrane is then placed on the surface of solid medium in a Petri dish. The method does not possess the disadvantages of previous methods of culture in liquid media, and also appears to permit a quantitative estimate of the extent of bacteraemia. Tidwell & Gee(116) describe a modification of the above method which, while it requires more processing steps, is shorter and a greater amount of blood is filtered. Kuzdas & Morse(117) have described a selective medium which they designated 'W. medium', for the isolation of brucella organisms from contaminated materials such as aborted foetuses, milk, water, soil and dung. To a litre of Albimi Brucella agar are added the following: 6000 units polymyxin B sulphate, 100 mg. actidione, 25,000 units bacitracin, 15,000 units circulin and 1·4 mg. crystal violet. Incubation in an atmosphere of 10% CO_2 is necessary for the cultivation on this medium of both *Br. abortus* and *Br. suis*. Renoux(118) reports that the substitution of ethyl violet for crystal violet in the above medium permits a better growth of *Br. melitensis*. The growth of small numbers (5–60) of a fastidious CO_2-dependent strain of *Br. abortus* in five liquid and on four solid media has been studied by Huddleson(119). None of the liquid media showed visible growth after incubation for 72 hr., but growth was stimulated by the addition of sterile bacterial extract, aged blood serum or serum albumin even when the pH was not optimal. Huddleson also reports(120) that two agar media which failed to support growth of a CO_2-dependent strain of type II *Br. abortus* became highly satisfactory when a sufficient number of killed cells of *Br. suis*, *Br. bronchisepticus*, *Salmonella pullorum* or *Staph. aureus* were added. An extract from either sonically disintegrated brucella or *Staph. aureus* contains the growth-promoting agent. The method of sonic disintegration has been described by the same author in a separate paper(121). Hess(122) describes a method for the serial testing of milk with special reference to cultural diagnosis of *Br. abortus* from cream samples.

Ortenzi(123) found that the diethylcarbamate test for the differentiation of brucella described by Renoux and reported in the previous review of this series(7) gave very pronounced growth with *Br. suis* and lesser and different degrees of inhibition with *Br. melitensis* and *Br. abortus*. He states that the method is highly reliable. Gargani & Donnini(124) and Bendtsen(125) have used this test in conjunction with the conventional methods of dye tolerance and production of H_2S to differentiate brucella species. Using a 'carrot-liver' medium and a modification of the diethylcarbamate test Negri & von Lorch(126) succeeded in typing fifty-six out of sixty-one strains of brucella. On the other hand, Bruni & de Felip(127) consider that the test is difficult to

interpret and that it is no improvement on existing methods. Cruickshank(128), however, is of the opinion that the pattern of growth and inhibition as it appears in the carbamate test as described by Renoux is of some value in the differentiation of the species. He also found that the three species differ in their sensitivity to 8-hydroxyquinoline(oxine), and that strains of *Br. suis* were more resistant to it than the other species. This observation may be of use in the identification of *Br. suis*. After examining 102 strains of the three species of brucella, Nyiredy(129) found that *Br. suis* was more sensitive to bile and produced more catalase than the other two species. He is of the opinion that *Br. abortus* and *Br. suis* are merely variants of *Br. melitensis*.

It is reported by Braun & Oglesby(130) that dye-resistant mutants of *Br. abortus* and *Br. suis* occurred spontaneously at an average rate of 6×10^{-10}. Most of the dye-resistant mutants and also mutants displaying altered CO_2 requirements competed successfully with their parent type when inoculated into guinea-pigs. It is not surprising therefore that an increasing number of aberrant strains are being found, especially in view of the increased attention that is being paid to the characterization of naturally occurring strains. Berman, Redfern & Simon(131) have found that colonial variants of *Br. abortus* and *Br. suis* establish themselves at certain times in the tissues of guinea-pigs inoculated with smooth cultures. Variants of *Br. abortus* have been recovered from the fluids of aborted bovine foetuses but not from the milk of aborting cows. Studies so far have been made on variants occurring in synthetic broth cultures and information regarding variants occurring *in vivo* is scanty.

A nitrite toxicity test has been developed by Pickett & Nelson(132) using tablets containing 2% nitrite. Of twenty-nine typical *Br. melitensis* twenty-six were sensitive to nitrite, whereas of twenty typical *Br. abortus* and sixteen typical *Br. suis* none were sensitive. The same authors(133) have also developed a method for performing qualitative carbohydrate fermentation tests. *Br. abortus* fermented inositol, mannitol (forty of forty-four strains) and rhamnose; *Br. suis* fermented maltose, mannitol and trehalose, whereas *Br. melitensis* fermented none of these carbohydrates. Using safranin or congo-red in a medium containing 1% glucose and lauryl sulphonate, Romváry(134) found there was a marked difference in dye-resistance in eight cultures of *Br. abortus*, strain 19, from various sources. Kraft(135) describes a simple diagnostic method for differentiating between cultures of *Br. abortus* and *Br. abortus* strain 19 by penicillin tolerance. Of forty-three cultures of strain 19, forty-one failed to grow in medium containing 1 unit/ml. of penicillin in air or 2 units/ml. in CO_2, whereas cultures of virulent *Br. abortus* grew in a concentration of 25 units/ml. A method used in producing type-specific brucella sera by quantitative agglutinin absorption is described by Roots & Sprockhoff(136). They state that *Br. melitensis* can be differentiated readily from *Br. abortus* and *Br. suis* if the absorption is done strictly quantitatively. Huddleson & White(137) report the isolation for the first time from the milk of infected dairy cows in Michigan of *Br. abortus* Type II (Wilson). Strains of this type show the following characteristics: they are dependent on CO_2 for growth, colonies on agar media are small, growth does not occur on agar media containing differential dyes or containing low concentrations of penicillin. Infection of the udder causes changes similar to those of mastitis.

Metabolic studies on the genus *Brucella* have been reported in the period under review. Cameron & Meyer(138), using conventional Warburg and paper chromatography techniques, have determined the comparative metabolic activity of washed cells of the three species of brucella when several different amino-acids that may participate in the urea cycle were used individually as substrates. *Br. suis* differed from the other two species in its oxidative and synthetic abilities, the greatest differences occurring with L-glutamic acid and D-alanine. Using the same methods the same authors(139) have shown also that resting cells of the three species of brucella metabolize urea, the chief amino-acids synthesized from it being alanine and glutamic acid. The same authors(140), studying the mechanism of differential bacteriostasis, examined the effect of basic fuchsin and thionin on the genus *Brucella*. They found that both dyes depressed the rate of respiration of the resting cell in *Br. abortus* and *Br. suis*, whereas only basic fuchsin depressed the rate in *Br. melitensis*. Altenbern & Ginoza(141) report that smooth cells of *Br. abortus*, strain 19, possess an enzyme which couples pantoyl lactone and β-alanine to produce

pantothenic acid. Evidence is presented by Mika, Braun, Ciaccio & Goodlow (142) of a relationship between the synthesis or utilization of pantothenic acid and the inhibitory effects of D-alanine or DL-alanine upon smooth cells in growing *Br. abortus* cultures involving the establishment of alanine resistant mutants. Studying the fixation of $C^{14}O_2$ in nucleic acid constituents by *Br. abortus*, strain 6232, growing on complex media Newton, Marr & Wilson (143) found that in this organism the only products of CO_2 fixation are pyrimidines. Further work (144) has shown that in strains 6232 and 6232C, the latter of which is capable of growth in air, the C^{14} from adenine is incorporated into the nucleic acid purines, adenine and guanine and that C^{14} from uracil is likewise incorporated into the pyramidine moieties. The organism is, however, incapable of interconverting purines and pyramidines.

C. *Vaccination*

In a previous review of this series (98) details were given of the immunity in cattle produced by inoculating them with 5·0 ml. of strain 19 vaccine subcutaneously and 0·2 ml. intradermally. Reporting on a continuation of this work McDiarmid (145) states that 0·2 ml. of strain 19 vaccine given subcutaneously is in no way inferior to the same dose given intradermally or to the larger dose (5·0 ml.) subcutaneously. The protective power of the vaccine was not so good as in the earlier experiments, but that may have been due to one or more of three factors: (1) that in the later experiments there was a shorter time between vaccination and challenge, (2) that the challenge dose was excessive, or (3) that pregnant heifers were used. An important point brought out by this work is that vaccination of pregnant cattle can be done without undue risk of it causing abortion, and this fact can be of great value when dealing with 'abortion storms' in non-vaccinated herds. A comparison of the effects of subcutaneous and intracaudal vaccination with strain 19 of sexually mature cattle is reported by Berman, Beach & Irwin (146). They found no significant difference in agglutinin response to vaccination with 0·25 ml. intracaudally and 5·0 ml. subcutaneously. Animals vaccinated by each route, when challenged by 12×10^6 organisms of *Br. abortus*, strain 2308, conjunctivally in the third gestation showed a considerable degree of immunity compared with the unvaccinated controls. Animals vaccinated as adults, or vaccinated as calves and revaccinated as adults have a greater tendency to develop agglutinins above the diagnostic level than animals vaccinated only as calves. The authors consider therefore that the use of strain 19 on sexually mature animals should be subject to strict limitations. An attempt to reinforce immunity to brucellosis created by calf-hood vaccination with strain 19 has been made by Gilman & Hughes (147). One month after conception in the third pregnancy 2·5 ml. of ether-killed vaccine was injected into a group of eleven cows and repeated when they were 3 months pregnant. A group of ten animals was left untreated. When animals of both groups that were 4 months pregnant were challenged with 6×10^6 organisms of *Br. abortus*, strain 2308 conjunctivally, a dose which caused two out of three control animals not vaccinated in calf-hood to abort, three animals in each group aborted showing that there was no apparent ability to enhance the calf-hood vaccination with strain 19. Realizing that the animal vaccinated with strain 19, which attains the status of a persistent reactor to the blood-serum-agglutination test, presents one of the more serious problems in the control and eradication of brucellosis, Morse, Robertstad, Beach & Ristic (148) obtained ten such animals from two brucellosis-free herds. The animals were slaughtered and various tissues and body fluids were taken for cultural examination and guinea-pig inoculation. Brucella organisms were not isolated from the bovine tissues or fluids or from the spleens of the guinea-pigs. By these laborious bacteriological studies the workers concerned could not detect any hazard to man or animals from any of the ten reactors. Gwatkin, Connell & Duthie (149) found no significant difference between the number of calves produced by a group of heifers vaccinated with one injection of ether-killed saline in oil suspension of *Br. abortus* and the number produced by unvaccinated controls following conjunctival challenge 1 month after vaccination, whereas a group vaccinated with strain 19 showed a difference in the number of living calves produced,

and the difference was just short of the conventional level of statistical significance. The figures given are: vaccination with killed-culture, 30·7% living calves produced, unvaccinated controls 35·7%, and strain 19 vaccinated 69·2%. Mantovani, Maglione & Ragni(150) found that calves born to cows vaccinated with strain 19 gave a positive reaction to the agglutination test in about 81% of the animals so treated. The antibodies were transferred via the colostrum and persisted for 4–20 days.

It has been pointed out by Scheer & Eisma(151) that only with a lyophilized vaccine can the guaranteed number of 60×10^9 living strain 19 organisms be attained. Lyophilized strain 19 may, however, contain a considerable number of organisms destroyed by drying which may provoke a strong degree of side reactions and this destruction should be kept to a minimum. Glycerol in a strength of 15–20% in normal saline solution has been found by Dzerzhinski(152) to be an excellent vehicle for live brucella vaccine. The organisms remained viable for 2 years and were stable even if exposed to severe frost or shaking in transport. Bergmann(153) discusses the advantage of freeze-dried over liquid strain 19 vaccine. He describes dilution techniques for determining the number of live *Br. abortus* as a means of testing the diluent in which the organisms are suspended during freeze-drying, and also for evaluating the stability of the vaccine and for testing the vacuum in sealed ampoules.

Comparative tests on the protective value of Huddleson's 'M'vaccine and strain 19 vaccine were reported in the previous review of this series(7). Further work has been described in the period now under review. Berman & Irwin(154) found that animals vaccinated with 'M' vaccine when they were calves showed no significant difference from unvaccinated control animals after exposure to 6×10^5 and 6×10^6 organisms of strain 2308 conjunctivally when $4\frac{1}{2}$–6 months pregnant, whereas those vaccinated with strain 19 were significantly more resistant as judged by incidence of abortion and by the number from which brucella were recovered from the lymph nodes after slaughter. Woods(155) reports that vaccination with 'M' vaccine of 1696 cattle in private herds in Illinois did not result in a persistent agglutination titre. A non-agglutinogenic strain, *Br. abortus* strain 'B' (Rosenbusch), has been compared with strain 19 by Sieiro(156). Both vaccines contained 10×10^9 organisms per ml. and a 5·0 ml. dose was given to each animal. Artificial exposure was made a year later to doses of 5×10^6 and 5×10^7 organisms. As the larger dose caused 100% abortions in the control animals it was considered excessive. The anti-abortion protective values for the lesser dose were as follows: strain 19 vaccinated 50%, strain 'B' vaccinated 47·6%, controls 18·2% and the anti-infectious values, strain 19 vaccinated 50%, strain 'B' vaccinated 19%. The author considers that strain 'B' vaccine, being non-agglutinogenic, could be used in sexually mature cattle.

Rolle(157) discusses suitable times and methods of vaccinating cattle in the light of the finding that immunity to *Br. abortus* infection depends on the resistance of the uterine mucosal cells, and that these cells can be stimulated earlier and more readily than other body cells. Isolation of 108 virulent strains of *Br. abortus* from the milk of cows vaccinated with strain 19 is reported by Sackmann(158). He is of the opinion that the infection of the udder was due either to a breakdown in immunity or to infection contracted before vaccination.

The method of assessment of brucellosis immunity in cattle at the Agricultural Research Council's Station at Compton has been given by Gordon(159). This involves the rearing of animals in a brucellosis-free environment, removing the animals to isolation buildings, and dividing them into vaccinated and control groups. At the fifth month of pregnancy a challenge dose is infused conjunctivally and at calving or at abortion tissues and milk samples are examined bacteriologically.

Immunization experiments on laboratory animals have also been reported during the period under review. McEwen & Samuel(160) have compared the effect of adjuvant on vaccination with three strains of *Br. abortus*. Liquid paraffin leve BP 4 parts and 'Falba' 1 part was used as the adjuvant. Challenge was by subcutaneous injection, made as far as possible from the original site of inoculation. Their first experiment showed that a heat-killed adjuvant vaccine made from a strain of low virulence for guinea-pigs (strain 45) affords as good or better protection than a living vaccine of the same strain in saline, and also that a heat-killed adjuvant vaccine made

from a non-agglutinogenic strain of low virulence (strain 45/20 McEwen) stimulates as high a resistance to infection as a heat-killed adjuvant vaccine made from a virulent strain (strain 544). Their second experiment showed that all of twenty animals given heat-killed adjuvant vaccine made from strain 45/20 resisted infection as shown by bacteriological examination of the spleen, whereas all of the twenty controls became infected. With heat-killed adjuvant vaccines made from both strain 45 and from strain 544 seven of twenty animals became infected. A heat-killed vaccine made from strain 544 suspended in saline protected only one of twenty animals. These experiments show that the antigen or antigens are heat-stable; antigenicity is enhanced by emulsification in oil, and antigenicity is not necessarily linked to the virulence or pathogenicity of the live organism. In the immunization of white rats against brucellosis Jacotot & Vallée (161) found that injection of dead vaccines was at least as efficient as injection of living vaccines. Similarly, Herzberg & Elberg (162) found no significant differences in the protection conferred on mice and guinea-pigs between injection of 10×10^9 living cells of a streptomycin-dependent strain of *Br. melitensis* and the response to the same number of cells killed by phenol, formaldehyde or heat. Streptomycin-resistant and non-resistant strains have been used by Olitzki & Sulitzeanu (163) in immunization experiments on white mice in order to distinguish between infecting and reinfecting organisms. They first demonstrated that streptomycin-resistant organisms did not revert to non-resistant organisms in white mice and vice versa. The optimal immunizing effect was obtained by using a quantity of living organisms sufficient to survive at the time of reinfection and capable of completely inhibiting the invasion of the reinfecting organism into the spleen. When the primary infecting strain, however, had disappeared from the infected organs at the time of reinfection there resulted a state of relative immunity in which the number of reinfecting organisms in the spleen remained limited and finally disappeared. Using a streptomycin-dependent strain of *Br. melitensis*, Elberg, Henderson, Herzberg & Peacock (164) made immunization experiments with Rhesus monkeys. They found no significant difference between immunity established by living or dead cells and concluded that the living cells did not survive in the tissues long enough to give more than a moderate degree of immunity.

D. *General*

Reports referring to the modes of spread of brucellosis have been published during the period under review. Kuzdas & Morse (165) artificially infected various materials including bovine urine, milk, water, dung and soil with *Br. abortus*, strain 2308. They found that temperatures of at or near freezing-point permitted survival for periods of over 824 days. Brucella organisms survived longer in carcasses left on the surface than in carcasses that were buried. Studying the role of rats as possible carriers of *Br. abortus* infection, Harms & Wegener (166) allowed laboratory rats to feed on cotyledons taken from the uteri of infected cows. Organisms were excreted in the rats' urine up to 36 hr. later, and they remained viable for about 24 hr. after the urine was voided. Noticing that brucella infection occurred in cattle which were grazing land that was heavily infected with ticks, Pritulin (167) suspected that they might be vectors of the infection. Engorged ticks, taken from four cows which had high titres to the complement-fixation test, were allowed to infest healthy guinea-pigs. The guinea-pigs became infected and material taken from them infected further guinea-pigs. Wellmann (168) confined some thousands of the common house-fly *Musca domestica* inside a building in which was placed the placenta of a cow with *Br. abortus* infection. After feeding on the placenta for a few hours the flies were allowed to fly into an adjacent building which housed eight pregnant heifers free from brucella infection. Four of the heifers became infected.

Work on experimental brucellosis in laboratory animals is reported. The infectivity of *Br. suis* for guinea-pigs by the respiratory route has been studied by Druett, Henderson & Peacock (169). The organism was dispersed in airborne particles of sizes varying from a single organism to particles that were 12μ in diameter. It was found that infectivity decreased 600-fold with increasing particle size within this range. A relationship between the natural pathogenicity of

a brucella organism and the persistence of the septicaemia it causes in guinea-pigs after subcutaneous inoculation has been found by Jacotot & Vallée(170). These authors(171) also describe a strain of *Br. abortus*, isolated from the milk of an infected cow, which was highly pathogenic for rabbits and guinea-pigs, intra-peritoneal injection of the organism causing severe purulent peritonitis. Mika, Goodlow, Victor & Braun(172) report that guinea-pigs exposed simultaneously to *Br. suis* and to sublethal doses of *Rickettsia burneti* developed both brucellosis and Q fever, though animals thus infected showed less severe illness than animals infected with *Br. suis* alone. Animals infected 11 or more days previously with *Br. suis* showed a significant resistance to lethal doses of *R. burneti*. This interference cannot be explained on the basis of demonstrable immunological factors. The glycoprotein content of serum was determined in guinea-pigs(173) infected with a virulent strain of *Br. suis* and treated with terramycin, ACTH, cortisone and 0·88% sodium chloride. Statistically significant increases were found in the infected animals irrespective of treatment.

Papers have been published on the subject of brucellosis in the human. Kronenwett, Lear & Metzger(174) report that thermal death times of *Br. abortus* in milk are such that the organism is certain to be killed under the conditions of pasteurization recommended by the U.S. Public Health Service. For example, pasteurization for 30 min. at 143° F. gives a margin of safety of approximately 26 min. Cruickshank(175) gives an account of 738 strains of brucella isolated in Britain and examined in the Brucella Reference Laboratory of the Public Health Laboratory Service. Of these, 680 were *Br. abortus*, of which sixty-two were of types that are inhibited by the usual dye tests. *Br. melitensis* (thirty strains) was identified from twenty-four farms and from one supply of bulk raw milk from tuberculin-tested cows. Clapp(176) states that only four doubtful cases of clinical or subclinical brucellosis occurred amongst forty-three people exposed to repeated infection by drinking unboiled milk containing *Br. abortus*. In contrast to findings in Buenos Aires reported previously(98), Maldonado(177) has observed that in the Municipal Abattoir of Santiago workers dealing with pigs were less often affected with brucellosis than those dealing with cattle, and of these latter the men dealing with cattle intestines were most often affected. Of 566 workers examined for brucella agglutinins only forty-three gave positive reactions. Various papers dealing with the risk run by veterinarians of becoming infected with brucellosis have been published. Paltrinieri(178) records that in Italy when a farm worker contracts brucellosis it is regarded as an 'accident at work', whereas there is no such provision for veterinarians in public service who are constantly exposed to infection. Herter(179) has published the results of a questionnaire sent to 3385 West German veterinarians. Brucellosis had been contracted by about 16% in the course of their work. According to Fejér(180) 14–16% of serum samples from veterinary personnel in Békés province in Hungary gave a positive reaction for brucellosis. A warning is given by Harris(181) that veterinarians should take all practicable precautions when handling animals whether vaccinated or unvaccinated. It has been found by Cedro, Cisale & Cacchione(182) that laboratory samples treated with formol for 3 hr. were quite safe to handle and their agglutination titres were unaffected.

Various drugs were tested by Hess(183) on cattle secreting *Br. abortus* in their milk. None had a lasting effect, though treatment with chloramphenicol was followed by a transitory reduction in the excretion of the organisms. Westring(184) found that the milk from infected cows which, prior to calving had 250 mg. of terramycin injected into each quarter of the udder, was free from *Br. abortus*.

E. *Control and eradication*

Three papers on the subject of brucellosis control and eradication were presented at the Annual Congress of the British Veterinary Association at Belfast in 1955. Mingle(185) reported that the incidence of brucellosis in the U.S.A. had been reduced from 11·5% in 1934, when the co-operative State-Federal programme was initiated, to 5% in 1955. The milk-ring test had been found to be most valuable as an effective means of economically screening large numbers of herds. He referred to the fact that Public Health aspects of brucellosis are playing an increasing role in

eradication. Nielsen(186) gave details of the 1948 Bill passed in Denmark for the control and eradication of brucellosis. The measures to prevent the spread of infection are of interest and include restrictions on cows which abort and control of bulls to be used on brucellosis-free herds. An owner of animals that are not free from the disease must prevent his stock from approaching nearer than 8 ft. of his neighbour's animals. The milk-ring test for screening purposes and blood tests are performed on herds showing infection. Herds are divided into three groups; those showing no reaction; those that react but have no clinical abortion and those in which clinical abortion occurs. Mitchell(187), discussing brucellosis control in Canada, considered that eradication could be done effectively if carried out on an area basis.

Referring to brucellosis control in Tasmania, Meldrum(188) states that at present a test and slaughter policy is adopted where the disease is found in a virtually clean area or where less than 10% of the herd is infected. Where incidence is high or more than 10% of the herd is infected, calves are vaccinated with strain 19. Brucellosis control in lightly affected areas in Australia is referred to by Stephens(189), who finds that the milk-ring test is of particular value in checking clean areas. Bennett(190) reports that for control in heavily infected areas in Australia reliance is placed on strain 19 vaccination of calves. While in general results have been good, in some herds the percentage of reactors has remained high even after 7 years of vaccination, and abortions still occur. He considers that slaughter of reactors is essential for the eradication of the disease. Discussing the control of brucellosis in Austria, Diernhofer(191) recommends a stricter interpretation of the agglutination test, and warns against accepting foreign agglutination-test certificates.

Surveying the subject of brucellosis in Great Britain, Robertson(192) points out that although many herds are still infected, the proportion of infected animals in each herd tends to be lower than it used to be. One infected animal in a herd, however, can be a danger to its fellows, and if it is giving infected milk it is a danger to the human population. He is of the opinion that strain 19, though a good vaccine, tends to engender an undue sense of complacency in the users. He suggests that in addition to vaccination, animals which react to the milk-ring test should be kept in isolation during their next calving period. This is sound advice, since it would appear from much of the work reported in the present review(99, 100, 166, 168) that the calving period is the time when infection is most liable to be spread. Stableforth(193) discussing brucellosis in man and animals states that in Great Britain strain 19 vaccination has reduced the incidence of abortion from about 9% to about 2% of all cows calving and most of the 2% is due to causes other than brucella. Excretion of brucella in the milk has also been reduced but not to the same extent. He expresses the hope that with regular vaccination the number of infected animals will continue to decrease and that a time will come when it will be possible to adopt measures for total eradication of brucella infection.

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